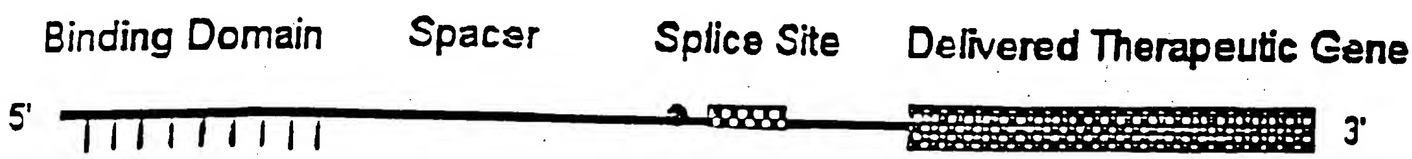
FIGURE 1A


Binding Domain
Spacer
Splice Site
Delivered Therapeutic Gene
5'
3'

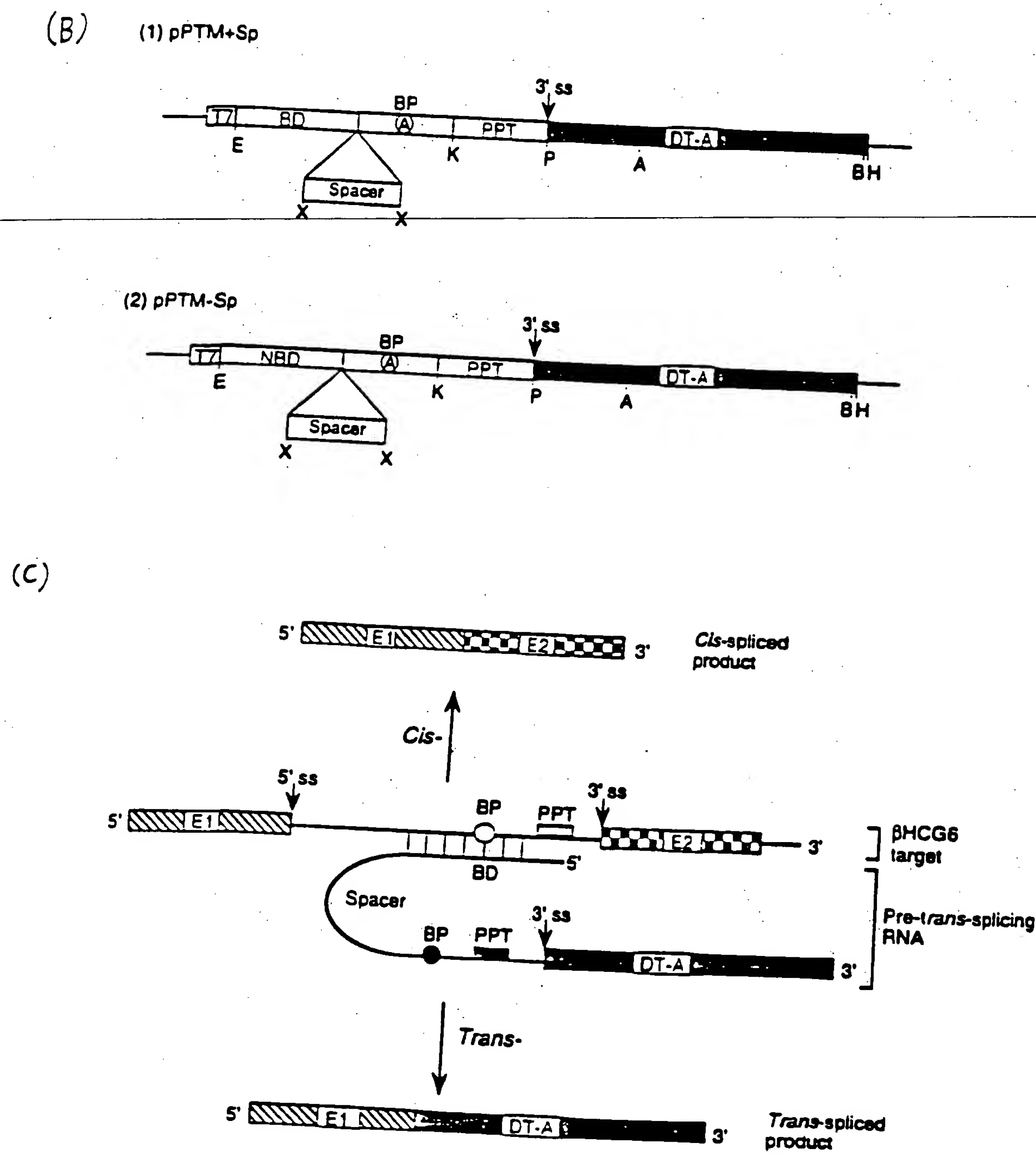
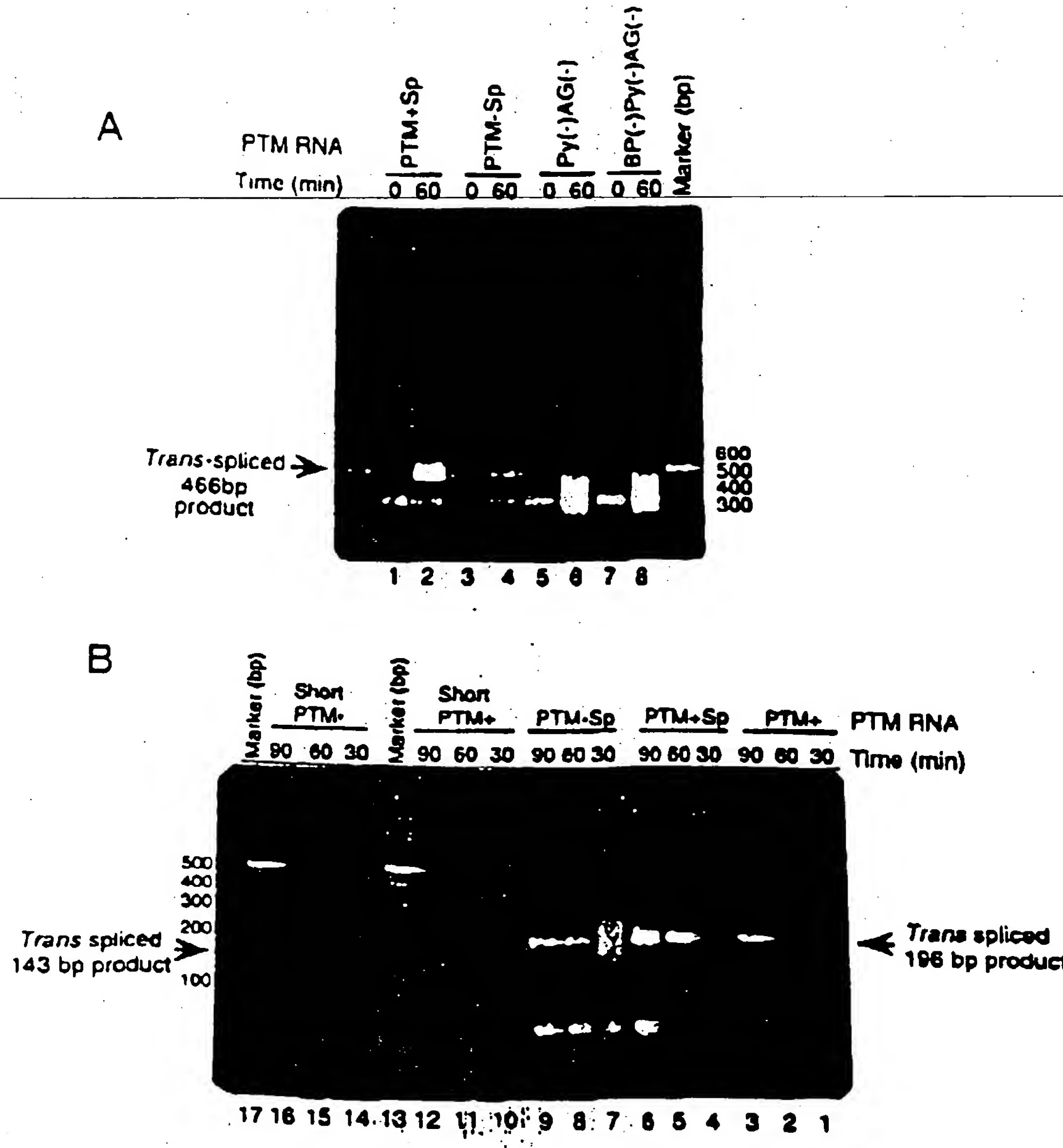
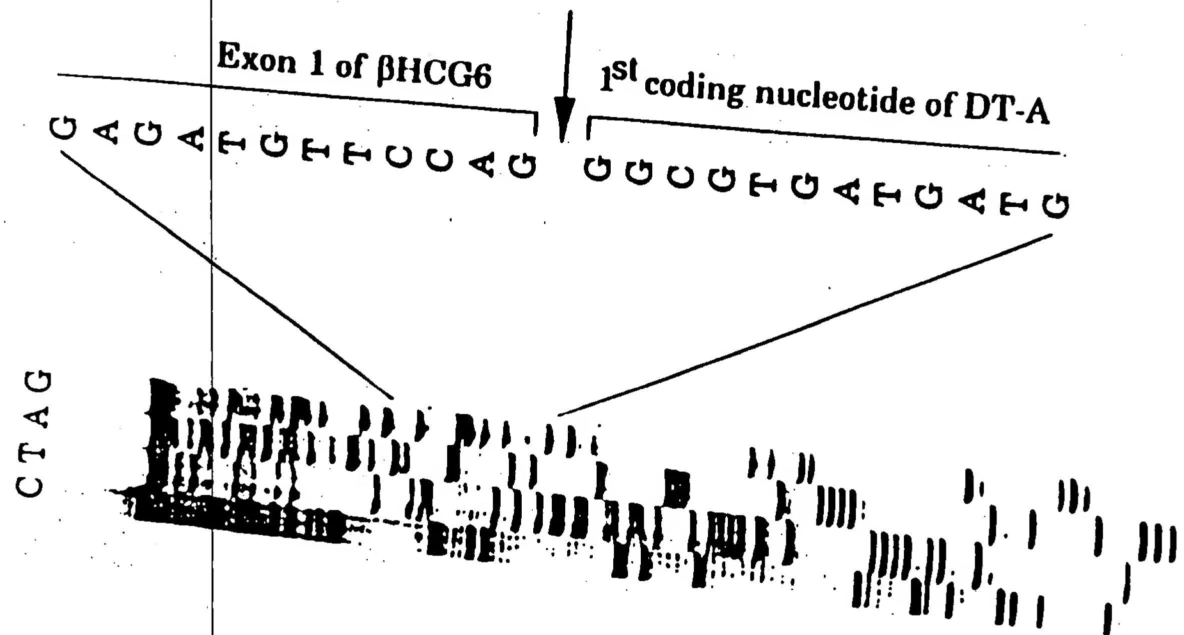
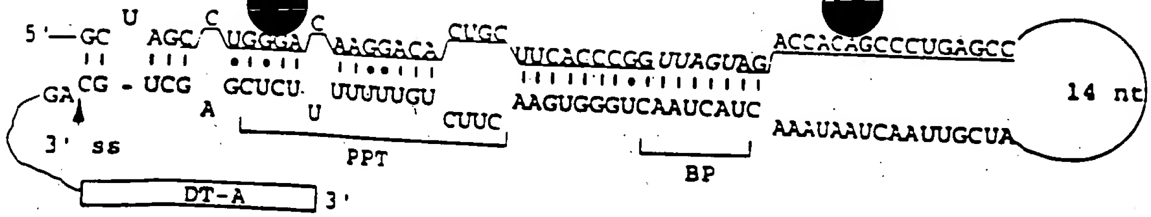

(B)
(1) pPTM+Sp
3' ss
BP
T7
BD
PPT
DT-A
E
K
P
A
BH
Spacer
X
X
(2) pPTM-Sp
3' ss
BP
T7
NBD
PPT
DT-A
E
K
P
A
BH
Spacer
X
X
(C)
5'
E1
E2
3'
Cis-spliced product
Cis-
5' ss
BP
PPT
3' ss
5'
E1
E2
3'
βHCG6 target
BD
5'
Spacer
3' ss
BP
PPT
DT-A
3'
Pre-*trans*-splicing RNA
Trans-
5'
E1
DT-A
3'
Trans-spliced product

Figure 1B-C

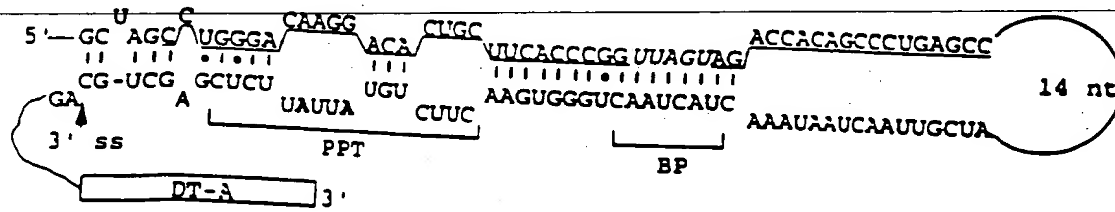

A
PTM RNA
Time (min)
PTM+Sp
PTM-Sp
Py(-)AG(-)
BP(-)Py(-)AG(-)
Marker (bp)
0 60
0 60
0 60
0 60
Trans-spliced 466bp product
600
500
400
300
1 2 3 4 5 6 7 8
B
Marker (bp)
Short PTM-
Marker (bp)
Short PTM+
PTM-Sp
PTM+Sp
PTM+
PTM RNA
90 60 30
90 60 30
90 60 30
90 60 30
90 60 30
Time (min)
500
400
300
200
100
Trans spliced 143 bp product
Trans spliced 196 bp product
17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1


Exon 1 of βHCG6
1st coding nucleotide of DT-A
G A G A T G T T C C A G
G G C G T G A T G A T G
C T A G

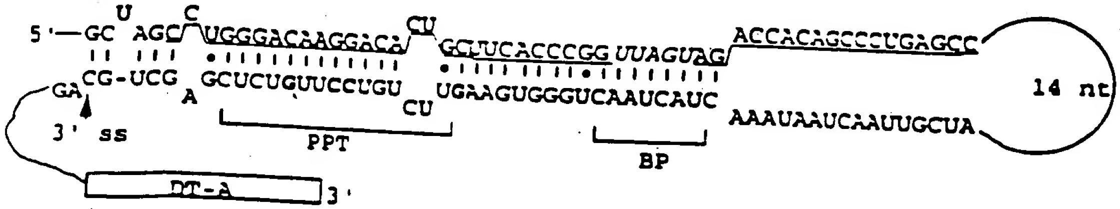
1. PTM+SF:



2. PTM+SF-Py1:



3. PTM+SF-Py2:



(B)

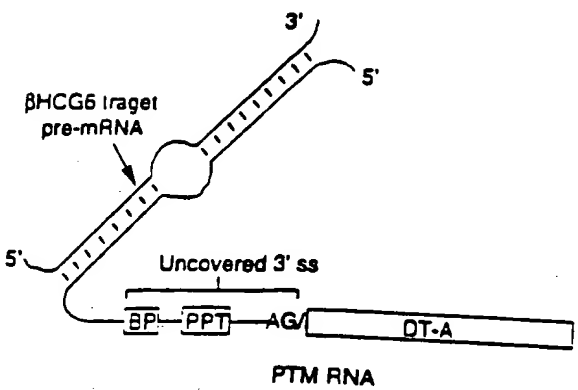


Figure 4A-B

(C)

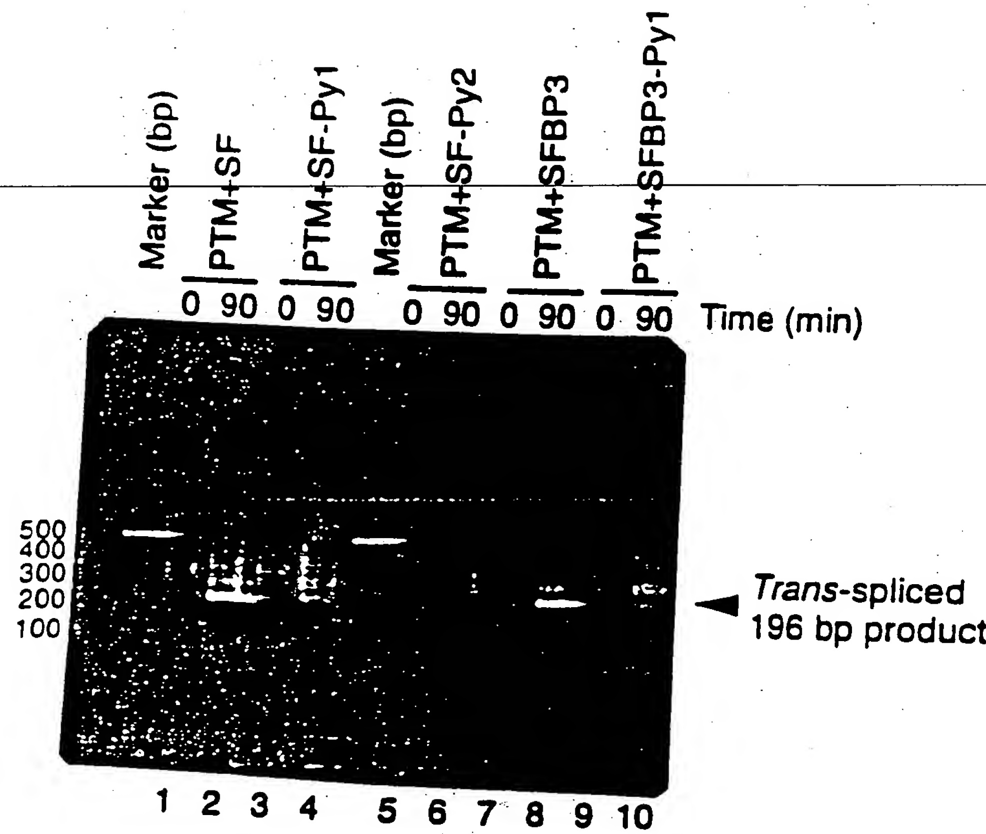

Marker (bp)
PTM+SF
PTM+SF-Py1
Marker (bp)
PTM+SF-Py2
PTM+SFBP3
PTM+SFBP3-Py1
0 90 0 90 0 90 0 90 0 90 Time (min)
500
400
300
200
100
Trans-spliced 196 bp product
1 2 3 4 5 6 7 8 9 10

Figure 4C

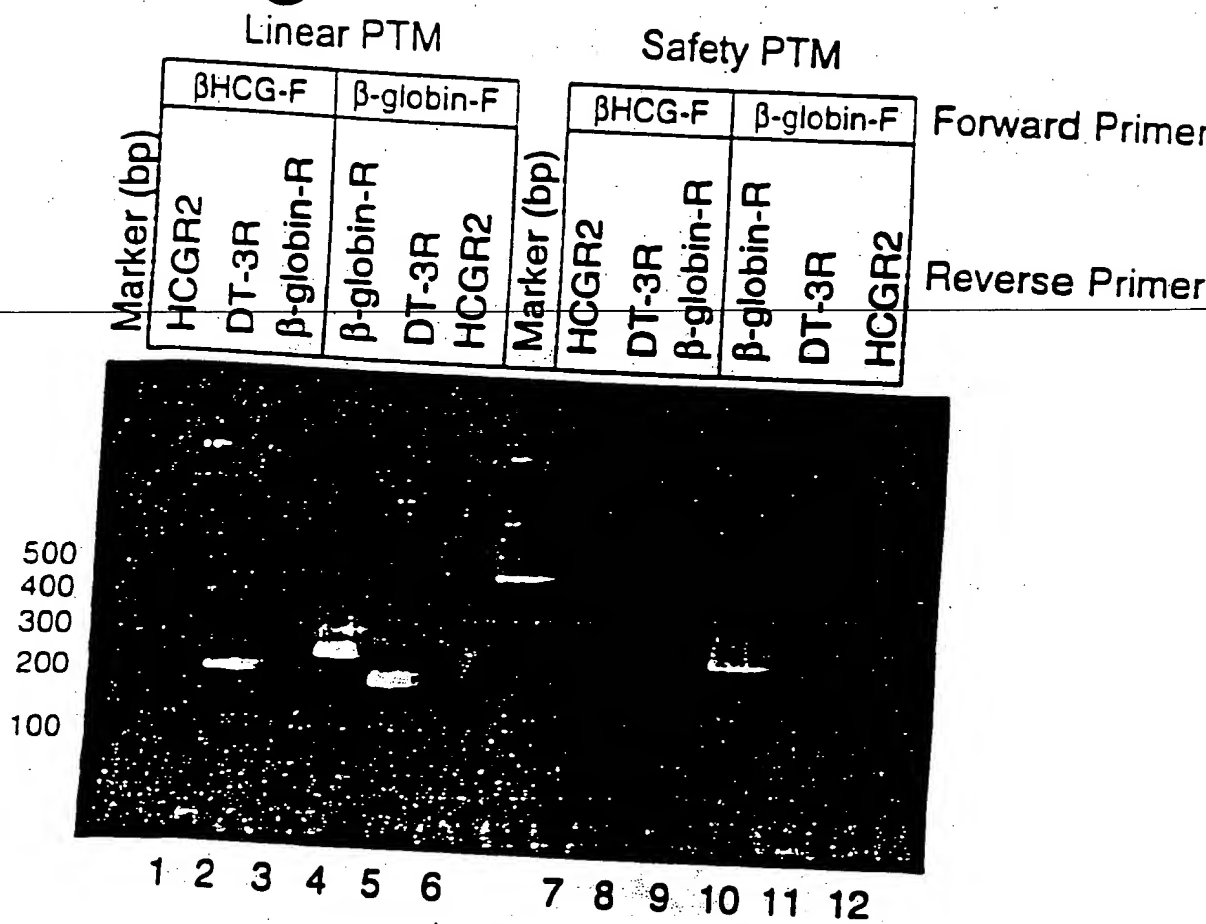

Linear PTM
Safety PTM
βHCG-F
β-globin-F
βHCG-F
β-globin-F
Forward Primer
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Reverse Primer
500
400
300
200
100
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5

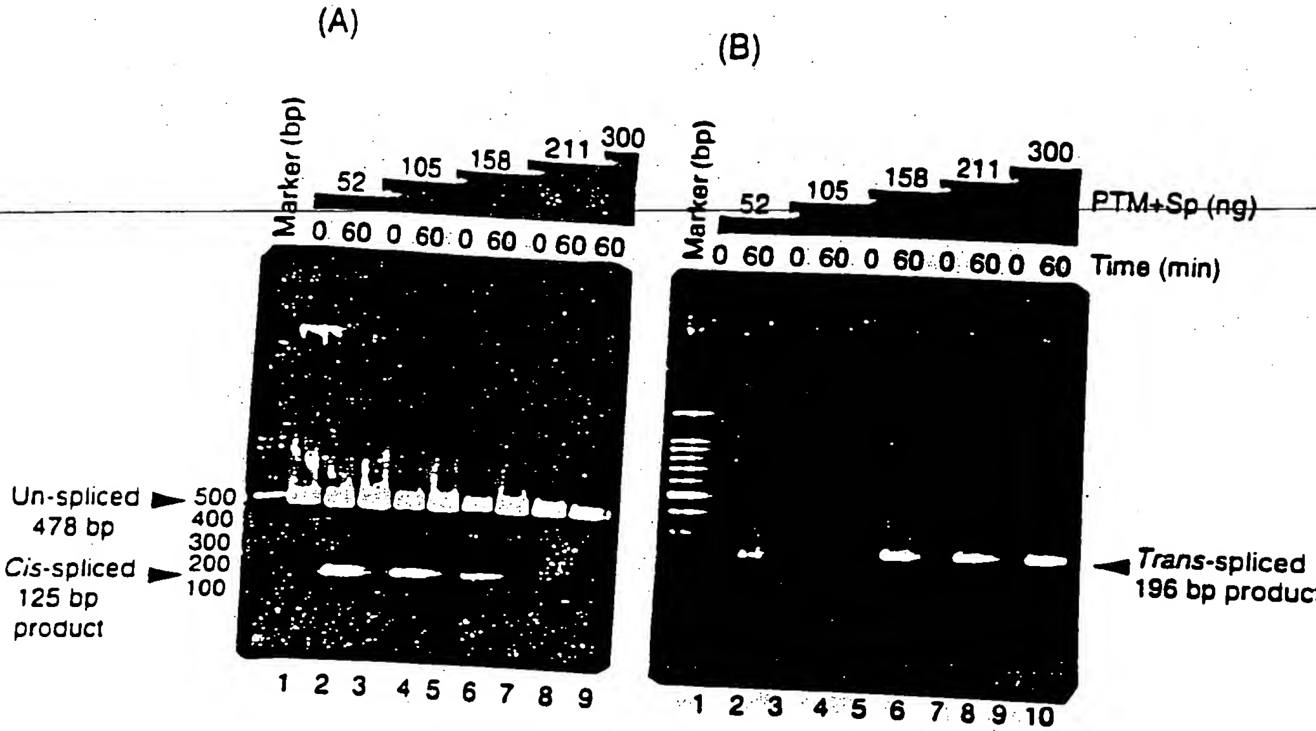
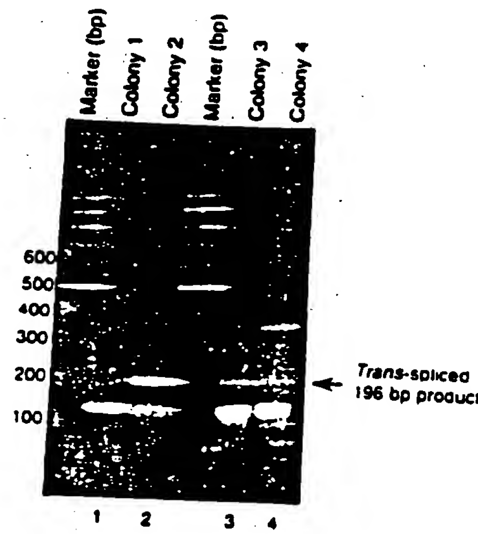

(A)
(B)
Marker (bp)
52
105
158
211
300
PTM+Sp (ng)
0 60 0 60 0 60 0 60 60
0 60 0 60 0 60 0 60 0 60
Time (min)
Un-spliced
478 bp
500
400
300
200
100
Cis-spliced
125 bp
product
Trans-spliced
196 bp product
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10

Figure 6

Figure 7

(A)



(B)

Exon 1 of βHCG6

5'-<u>CAGGGGACGCACCAAGGATGGAGATGTTCCAG</u>-GGCGCTGATGATGTTGTT

1st coding nucleotide of DT-A

GATTCTTCTTAAATCTTTTGTGATGGAAAACTTTTCTTCGTACCACGGGACTA

AACCTGGTTATGTAGATTCCATTCAAAAA-3'

Double Splicing Pre-therapeutic RNA

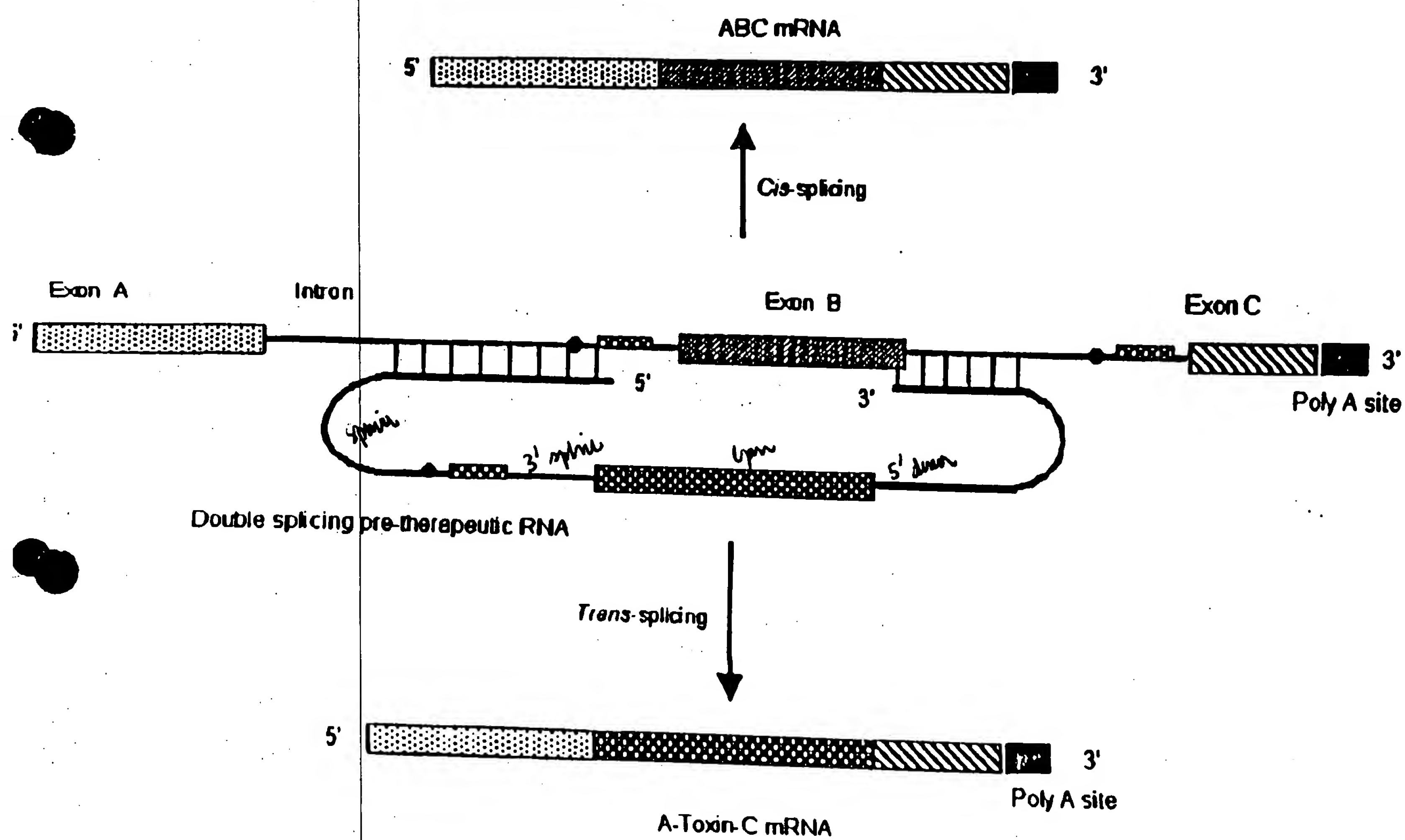
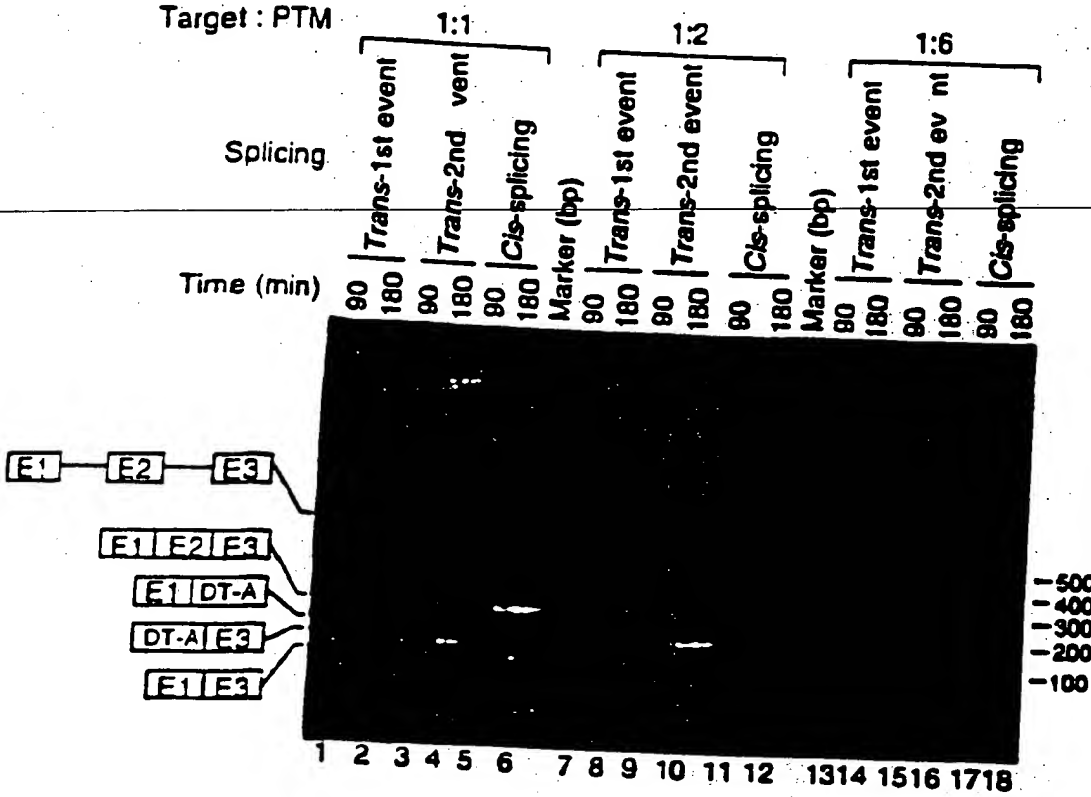


Figure 8A

31304B-A
(Sheet 10 of 66)

Selective Trans-splicing of a Double Splicing PTM

(3' ss of PTM to 5' ss target and, 5' ss of PTM to 3' ss of target)



Cis-spliced products

E1 E2 E3 = Normal *cis*-splicing (277bp)

E1 E3 = Exon skipping (110bp)

Trans-splicied products

E1 DT-A = 1st event, 196bp. *Trans*-splicing between 5' ss of target & 3' ss of PTM.

DT-A E3 = 2nd event, 161bp. *Trans*-splicing between 3' ss of target & 5' ss of PTM.

Figure 8B

31304B -A

(Sheet 11 Of 66)

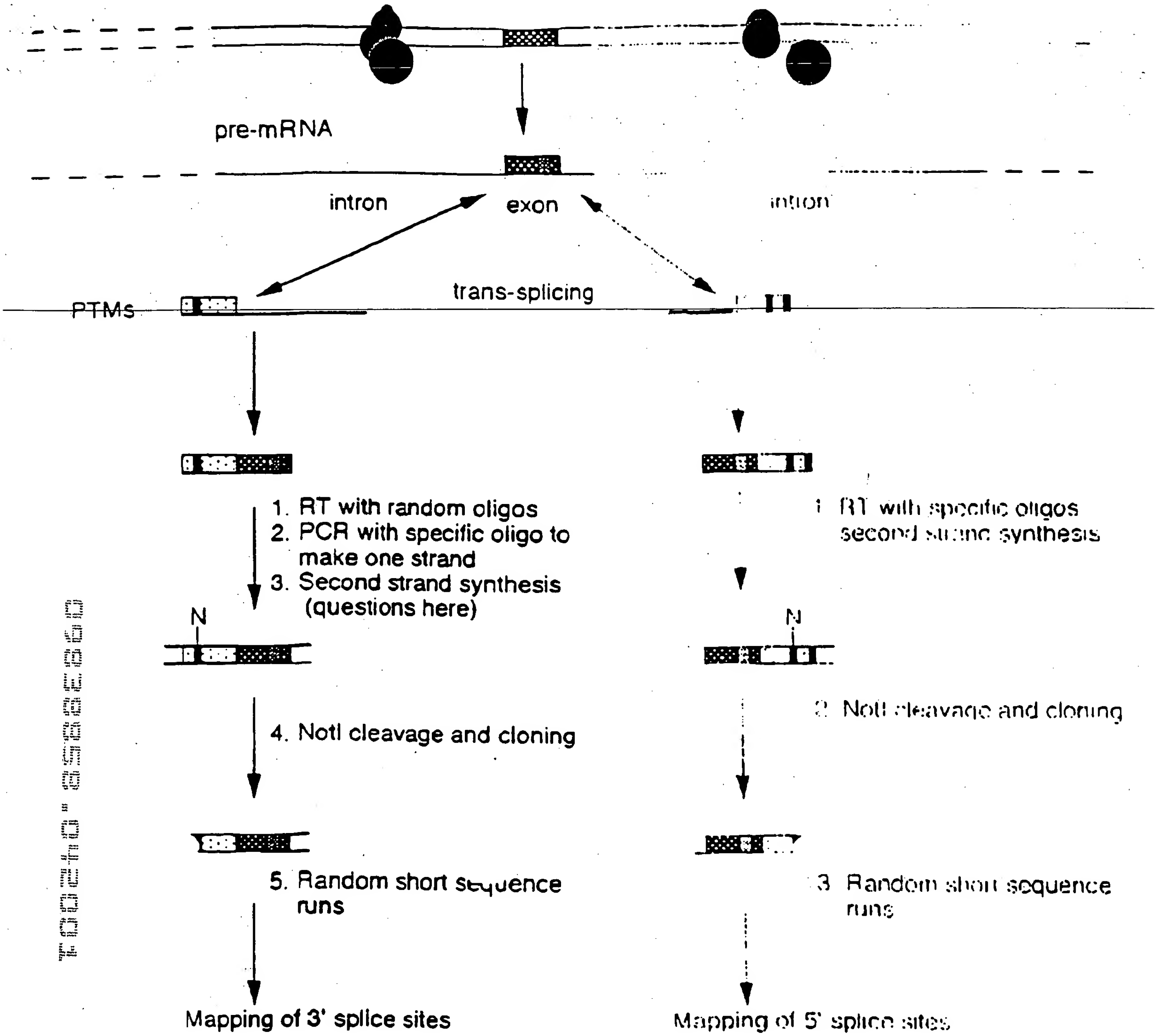


FIGURE 9

31304B-A
(Sheet 12 Of 66)

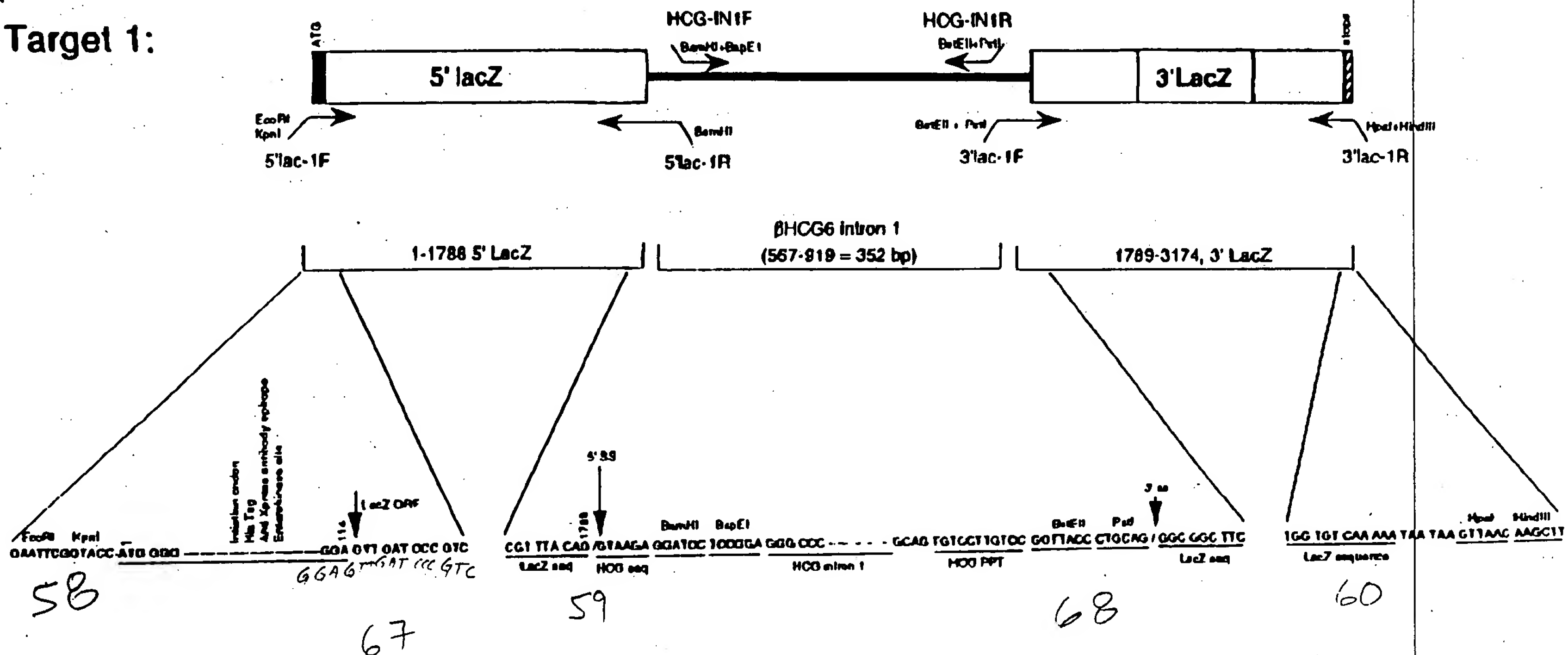
31 304 B-A (sheet 13 of 66)

FIG. 10 A

Knock Out LacZ Model Constructs

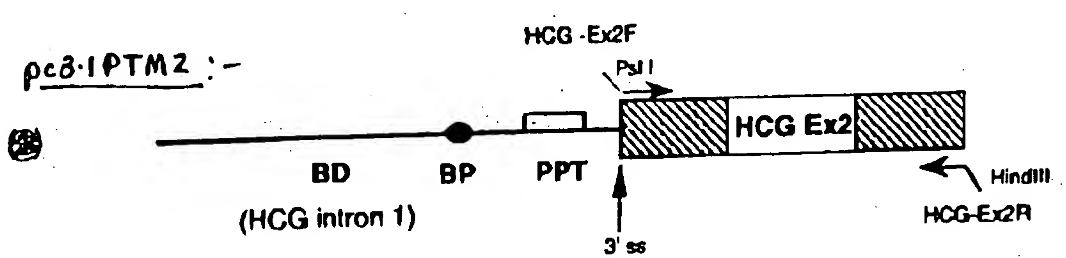
pc3.1 lac-T1

Target 1:



PTMs

pc3.1 PTM2 :-



Restoration of β-Gal activity by SMaRT

(Spliceosome Mediated RNA *Trans*-splicing)

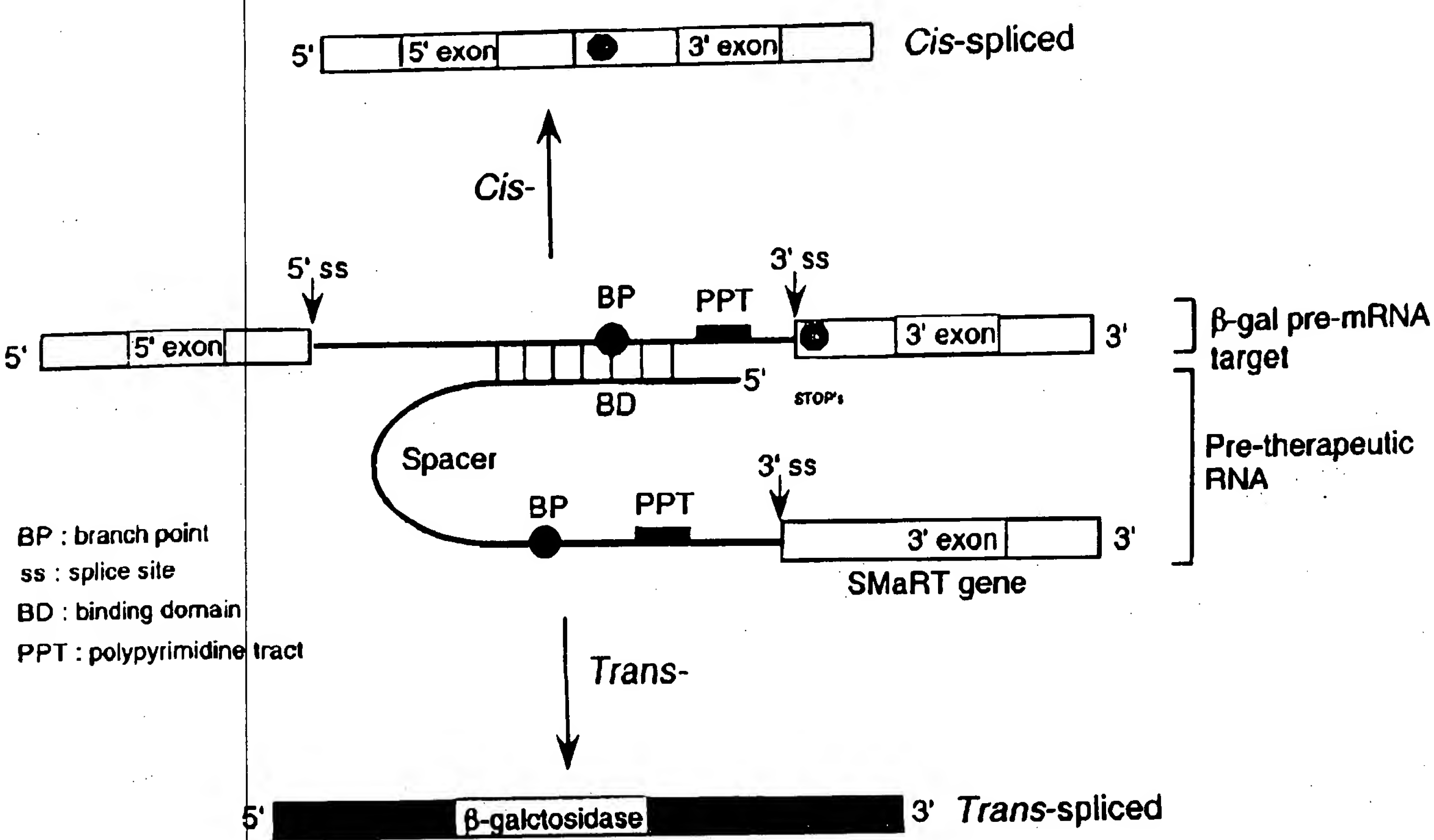


Figure 10B

31304 B-A
(Sheet 14 of 66)

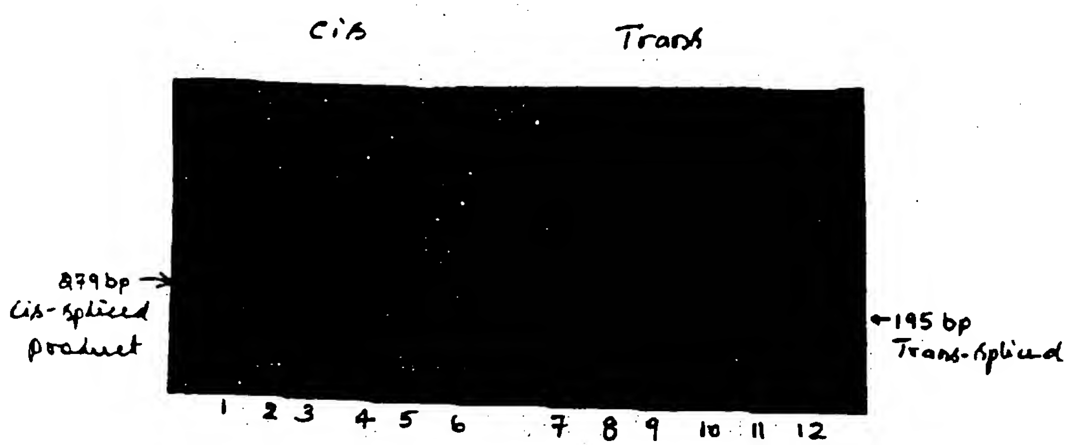

Cis
Trans
879 bp
Cis-spliced product
←195 bp
Trans-spliced
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 11A

Figure 11 B

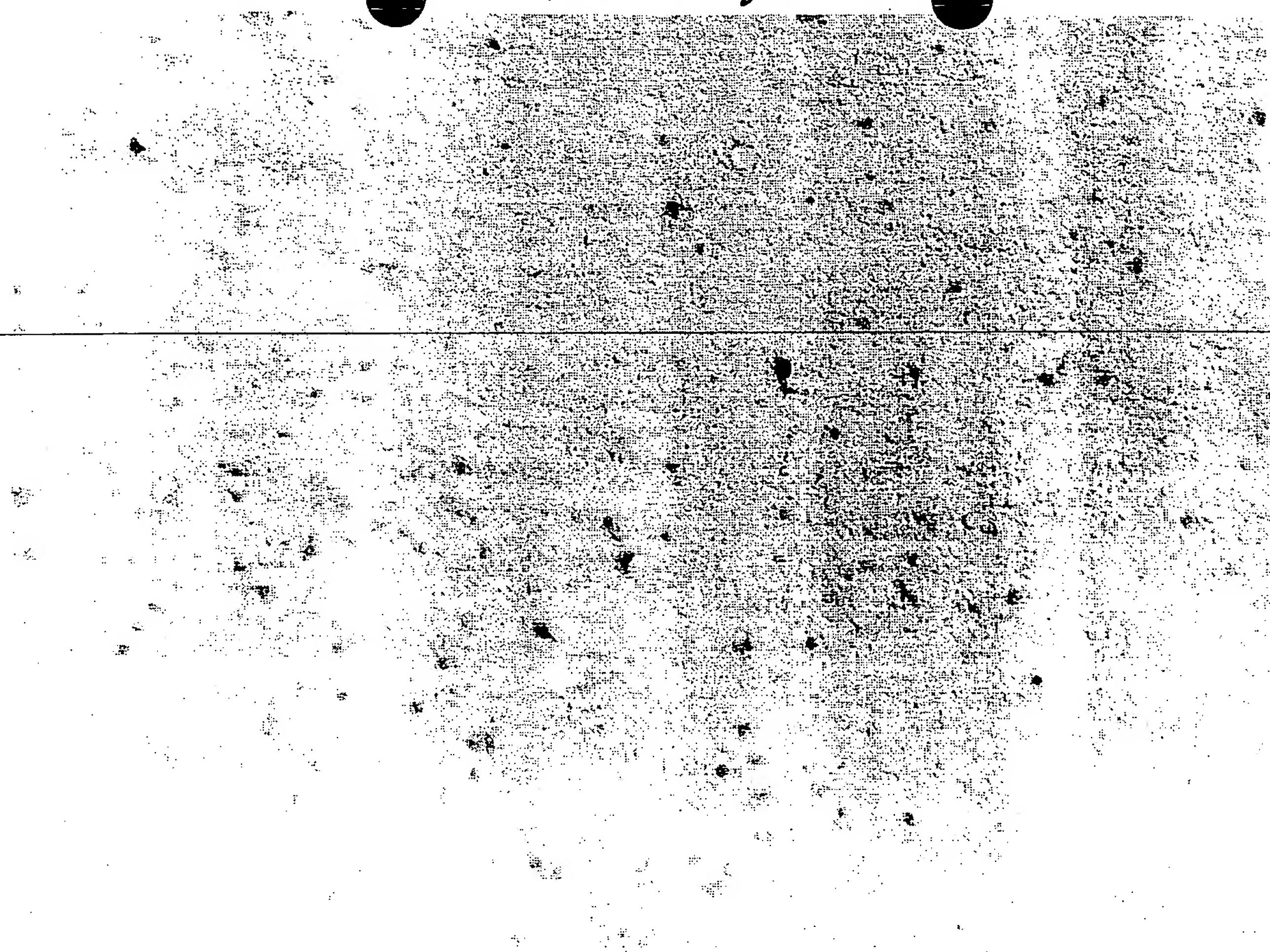


FIGURE 11C

Nucleotide Sequence Demonstrating that *Trans*-splicing is Accurate

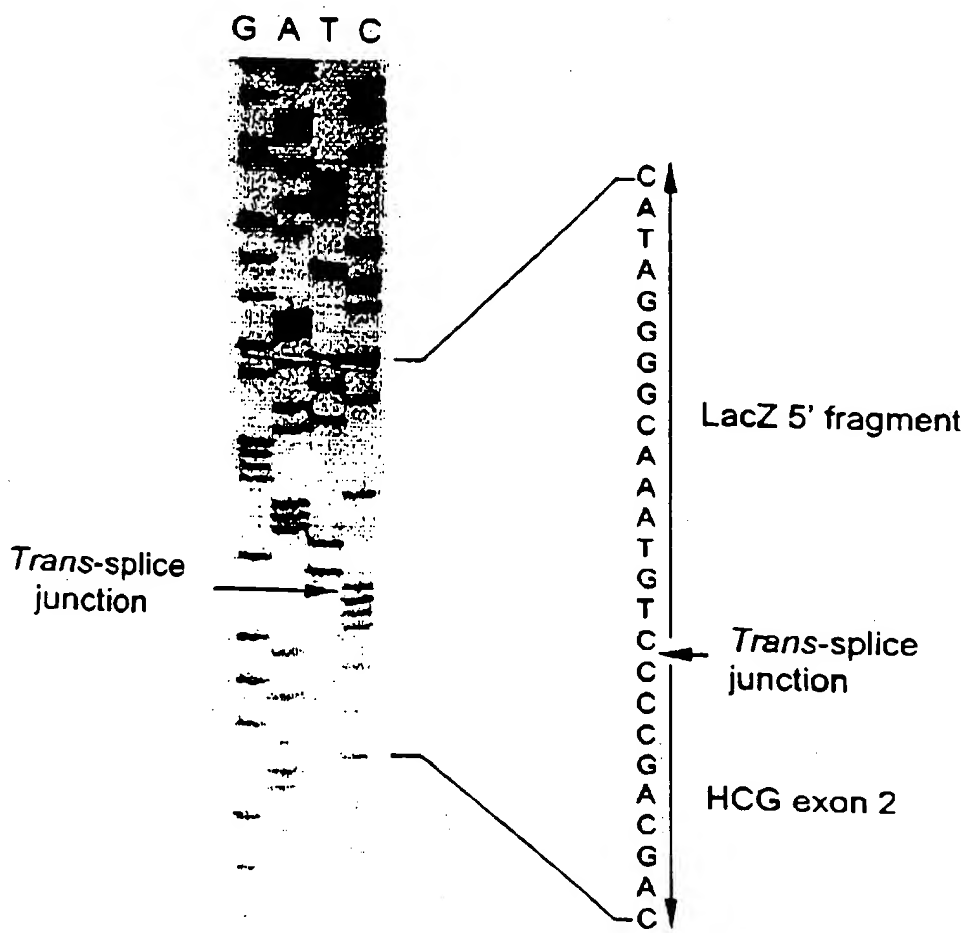


FIGURE 12 A

31304-B-A
(Sheet 18 of 66)

(1). <u>Nucleotide sequences of the *cis*-spliced product (285 bp)</u> :

BioLac-TR1
GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTG

Splice junction
GCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGCGGCTTCGTCTAAATAATG

GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTT

Lac-TR2
TGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGGCGACCGCACGCCGCATCCAG

(2) <u>Nucleotide sequences of the trans-spliced product (195 bp)</u>

BioLac-TR1
GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTGG

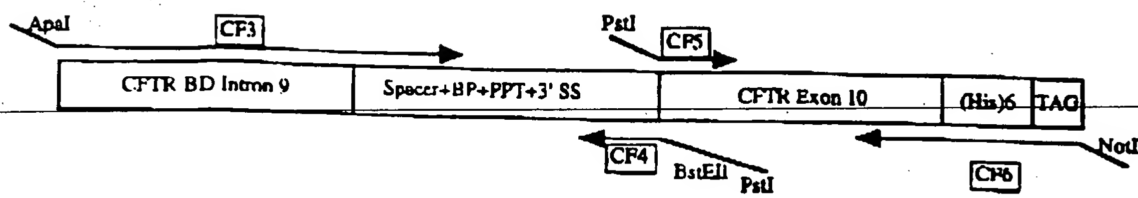
Splice junction
CGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGGCTGCTGCTGTTGCTGCTGCT

HCGR2
GAGCATGGGCGGGACATGGGCATCCAAGGAGCCACTTCGGCCACGGTGCCG

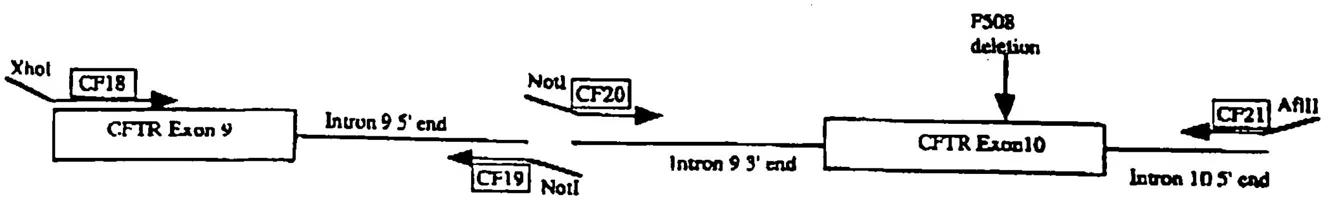
Figure 12 B

31304-B-A
(Sheet 19 of 66)

CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target - construction



TRANS- SPLICING Repair

Binding of PTM to TARGET

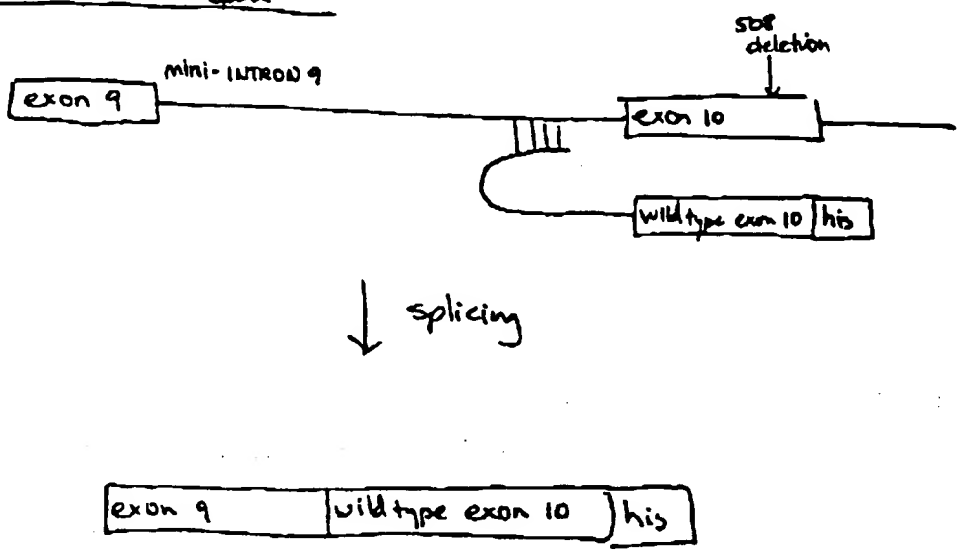


Figure 13

31304-B-A
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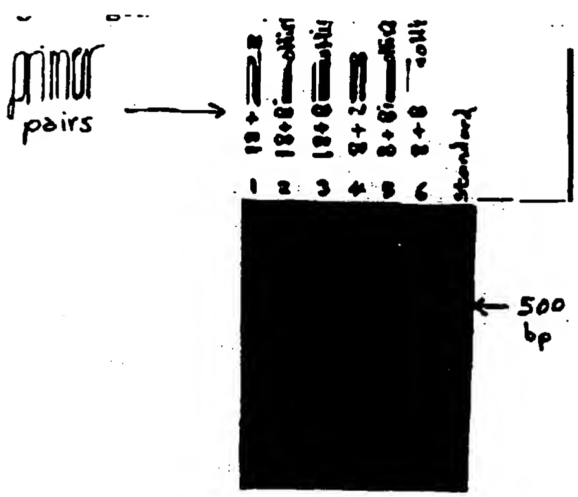
Figure 14



31304 B-A
(Sheet 21 of 66)

DNA sequence 500 b.p. GCTAGCGTTTAA ... TGCCACTCCCAC linear

Positions of Restriction Endonucleases sites (unique sites underlined)

```
                  Sau96 I
                  Hae III
                  Sau96 I
                  Ban II                    Binding domain
Nhe I    Dra I    Apa I     Intron 9 BD          Sac II                   Xho I  Sca I
GCTAGCGTTTAAACGGGCCCACCCATCATTATTAGGTCATTATCCGCGGAACATTATTATAACGTTGCTCGAGTACTAAC   80
CGATCGCAAATTTGCCCGGGTGGGTAGTAATAATCCAGTAATAGGCGCCTTGTAATAATATTGCAACGAGCTCATGATTG
1        8        15                          44                      68   72
                  15
                  15
                  16
                  16

Kpn I                 Pst I 3'ss         Exon 10 CFTR + His tag + STOP
TGGTACCTCTTCTTTTTTTTCCTGCAGACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAAT  160
ACCATGGAGAAGAAAAAAAAAGGACGTCTGAAGTGAAGATTACTACTAATACCCTCTTGACCTCGGAAGTCTCCCATTTTA
82                    102

         Xmn I           Dde I                                                F508
TAAGCACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTG  240
ATTCGTGTCACCTTCTTAAAGTAAGACAAGAGTCAAAAGGACCTAATACGGACCGTGGTAATTTCTTTTATAGTAGAAAC
         172             190

                                        Sph I                  His             STOP
GTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGCATCATCATCATCATCATTAG  320
CACAAAGGATACTACTTATATCTATGTCTTCGCAGTAGTTTCGTACGGTTGATCTTCTCGTAGTAGTAGTAGTAGTAATC
                                        282

                                                              Sac I
                                                              Ban II
                                                         Sau3A I
 Hae III                Pst I                            Dpn I
Not I            EcoR V     EcoR I                       BamH I        Kpn I   HinD III    Dra I
GCGGCCGCCACTGTGCTGGATATCTGCAGAATTCCACCACACTGGACTAGTGGATCCGAGCTCGGTACCAAGCTTAAGTT  400
CGCCGGCGGTGACACGACCTATAGACGTCTTAAGGTGGTGTGACCTGATCACCTAGGCTCGAGCCATGGTTCGAATTCAA
321              339        349               CF28 372              384    390          399
 323                    344                        373
                                                   373
                                                       378
                                                       378
```

Present in PTM 3'UT but not Target.

```
     Sau3A I
     Dpn I
TAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACC  480
ATTTGGCGACTAGTCGGAGCTGACACGGAAGATCAACGGTCGGTAGACAACAAACGGGGAGGGGGCACGGAAGGAACTGG
     410              CF27
     410

CTGGAAGGTGCCACTCCCAC   500
GACCTTCCACGGTGAGGGTG
```

~~63~~ 64 ~~64~~ ~~65~~ ~~65~~ ~~66~~

Restriction Endonucleases site usage

Acc I	-	EcoR I	1	Nde I	-	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
ApaL I	-	Hae II	-	Not I	1	Sma I	-
Avr II	-	Hae III	2	PflM I	-	Sph I	1
BamH I	1	HinC II	-	Pst I	2	Spl I	-
Ban II	2	HinD III	1	Pvu I	-	Ssp I	-
Bbe I	-	Hinf I	-	Pvu II	-	Stu I	-

31304-A-B
(Sheet 22 of 66

EXPERIMENT 2

Repair of an exogenously supplied CFTR target molecule carrying an F508 deletion in exon 10.

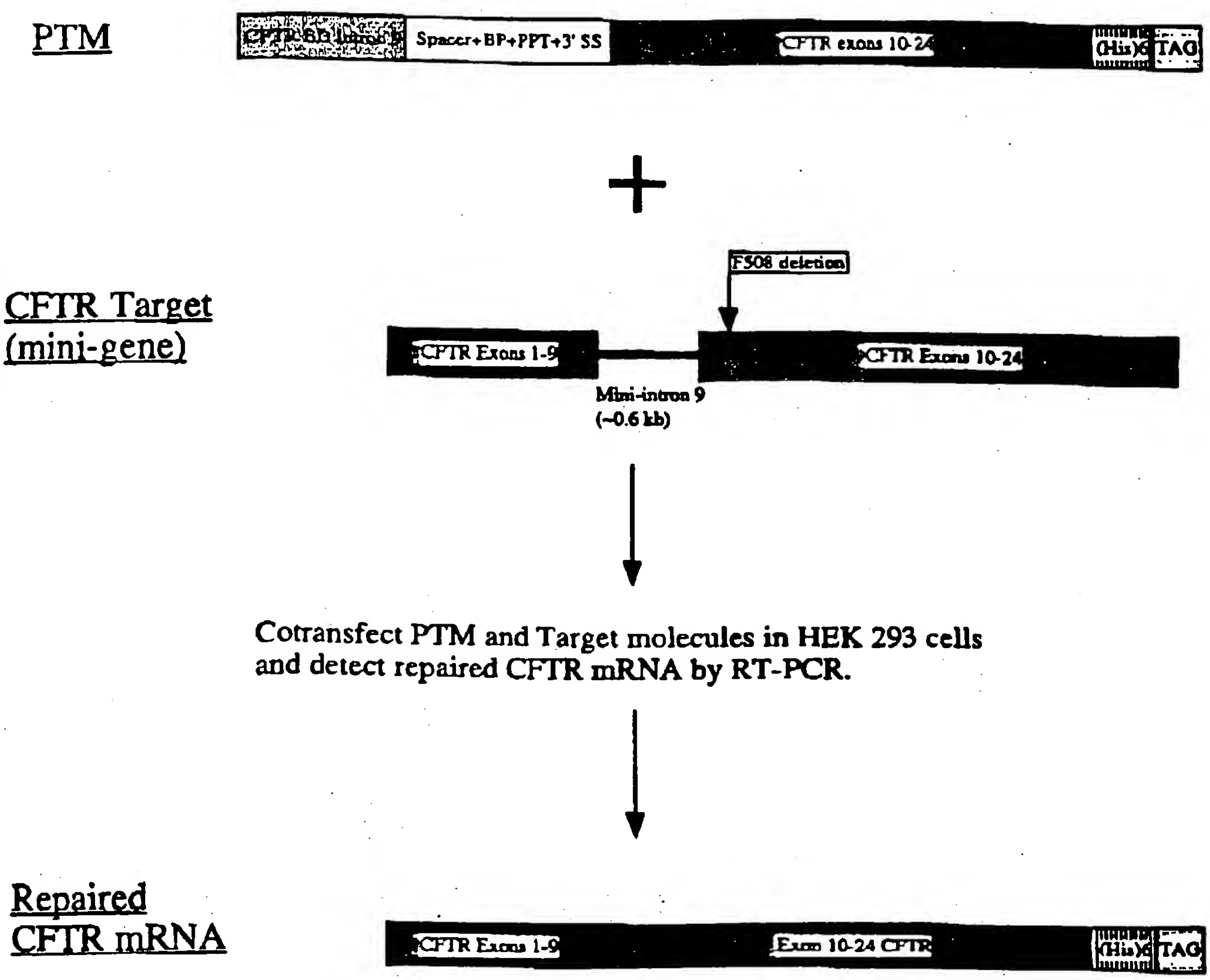


Figure 16

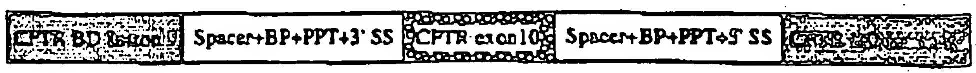
31304-A-B

Sheet 23 of 66)

EXPERIMENT 3

Repair of endogenous CFTR transcripts by exon 10 invasion using a double splicing PTM

Double Splicing PTM



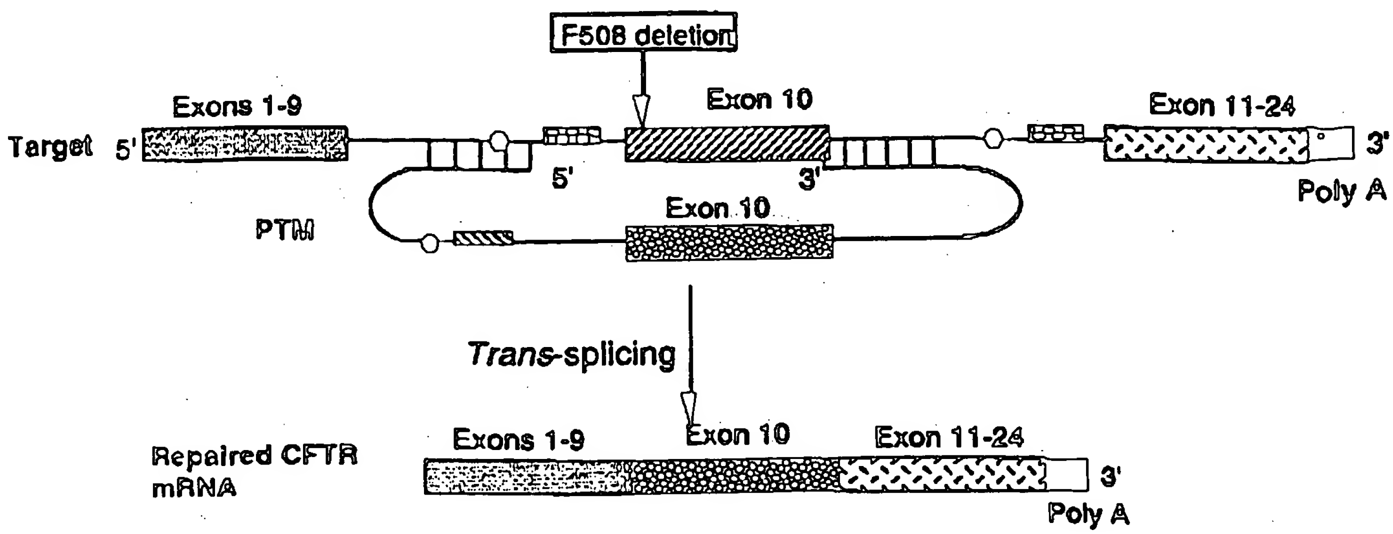


Figure 17

31304 B-A

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Double *Trans*-splicing Specific Target

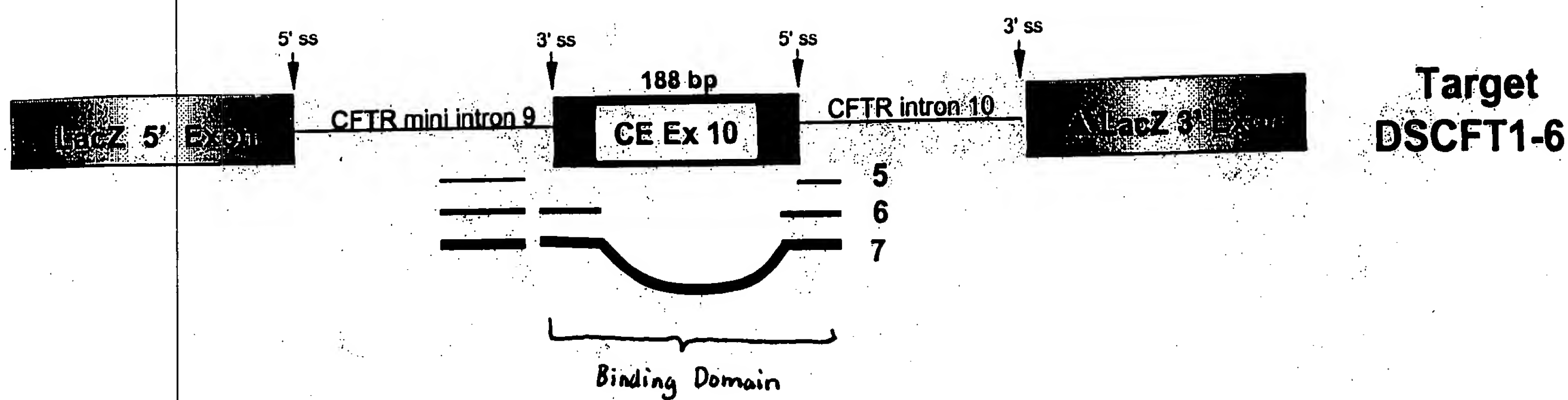

Figure 18

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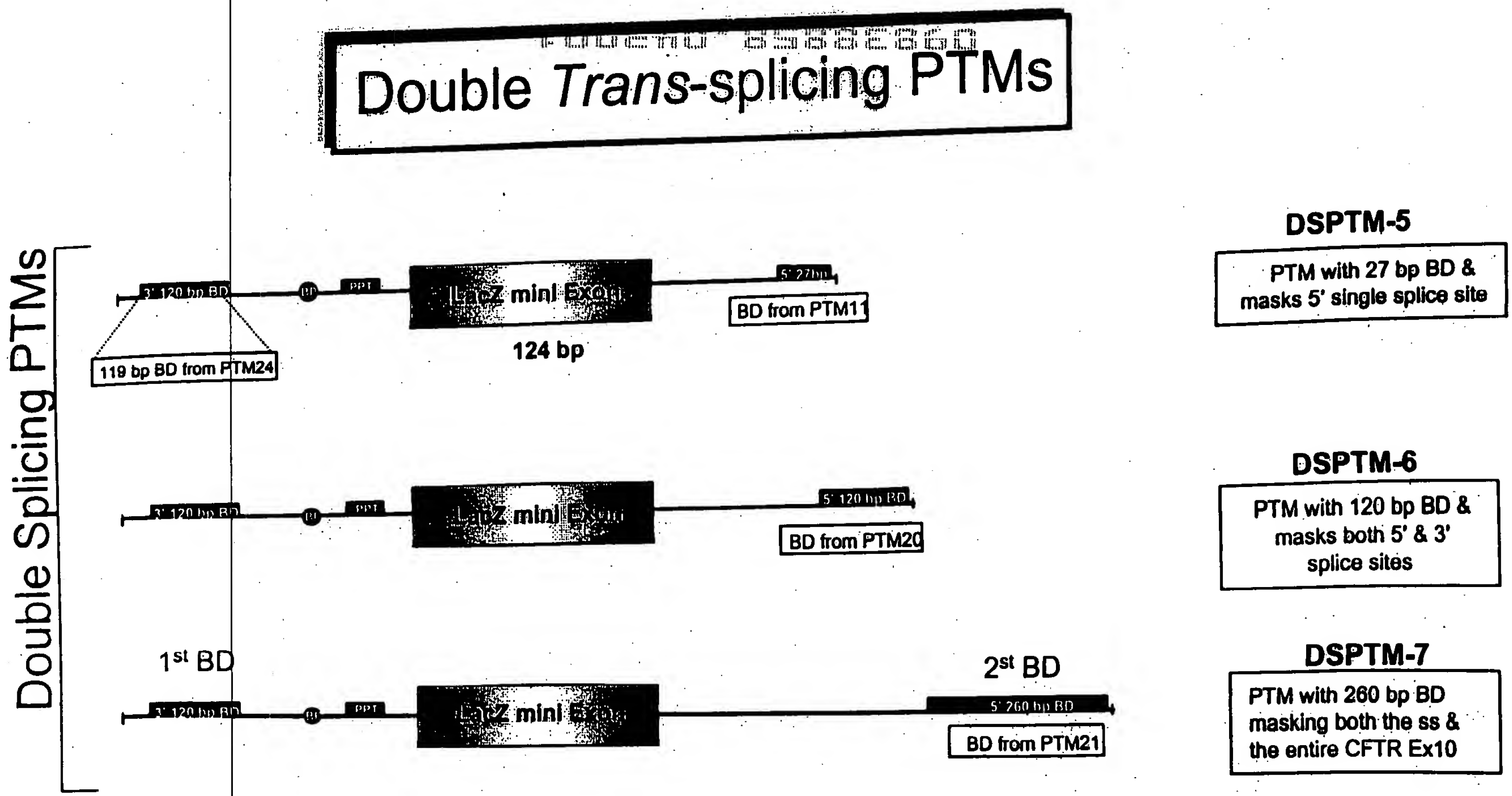

Figure 19

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Double *Trans*-splicing β-Gal Model

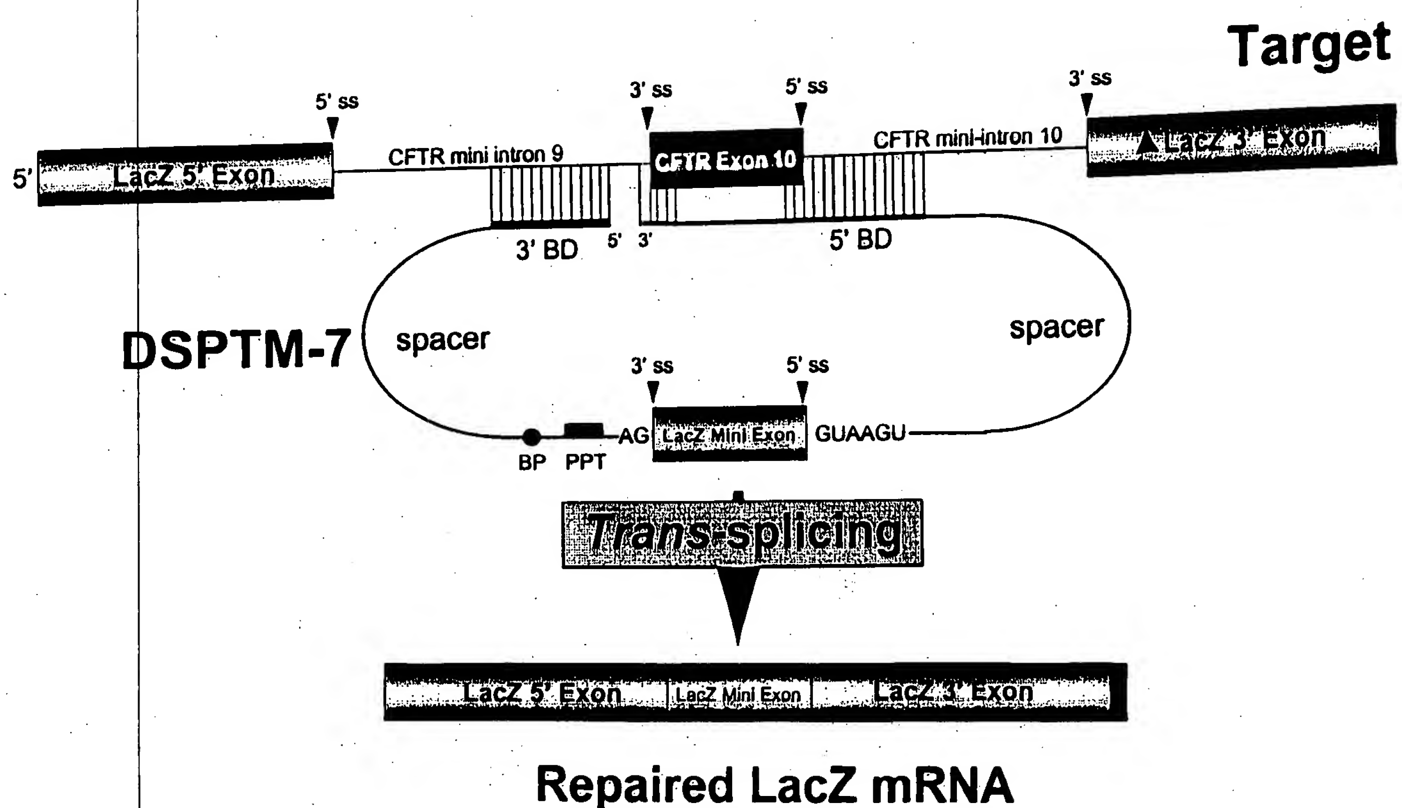
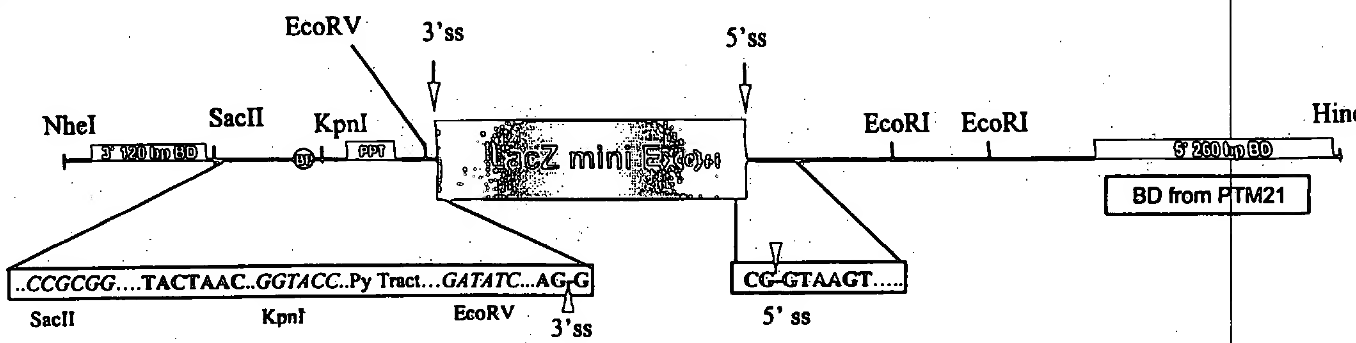


Figure 20

TOOZHO" 85882860

Important Structural Elements of DSPTM-7: (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



(1) 3' BD (120 BP) : GATTCACTTGCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTATTTGTAAAGATTCTATTAACTCATTTGATTC AAAATATTTAAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T *GGTACC* TCTTCTTTTTTTTTTT *GATATC* CTGCAG GGC GGC

(BP — Kpn I — PPT — EcoRV — 3' ss — LacZ mini exon)

(4) 5' donor site and 2nd spacer sequence: TGA ACG *GTAAGT* GTTATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACG CTAAGATCCACCGG

(LacZ mini exon — 5' ss)

(5) 5' BD (260 BP) : TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTT*GAATTC*ATGCTTTGATGACGCTTCTGTATCTATATTCATCATTGGAA ACACCAATGATTTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGTGCTTAA AAAAACCCTCT*GAATTC*TCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATTATTAACTCA TTATCAAATCACGC

Figure 21

DSPTM8 :(▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)

PTM29 (lacks 2nd BD and 5' ss)

PTM30 (lacks 1st BD and 3' ss)

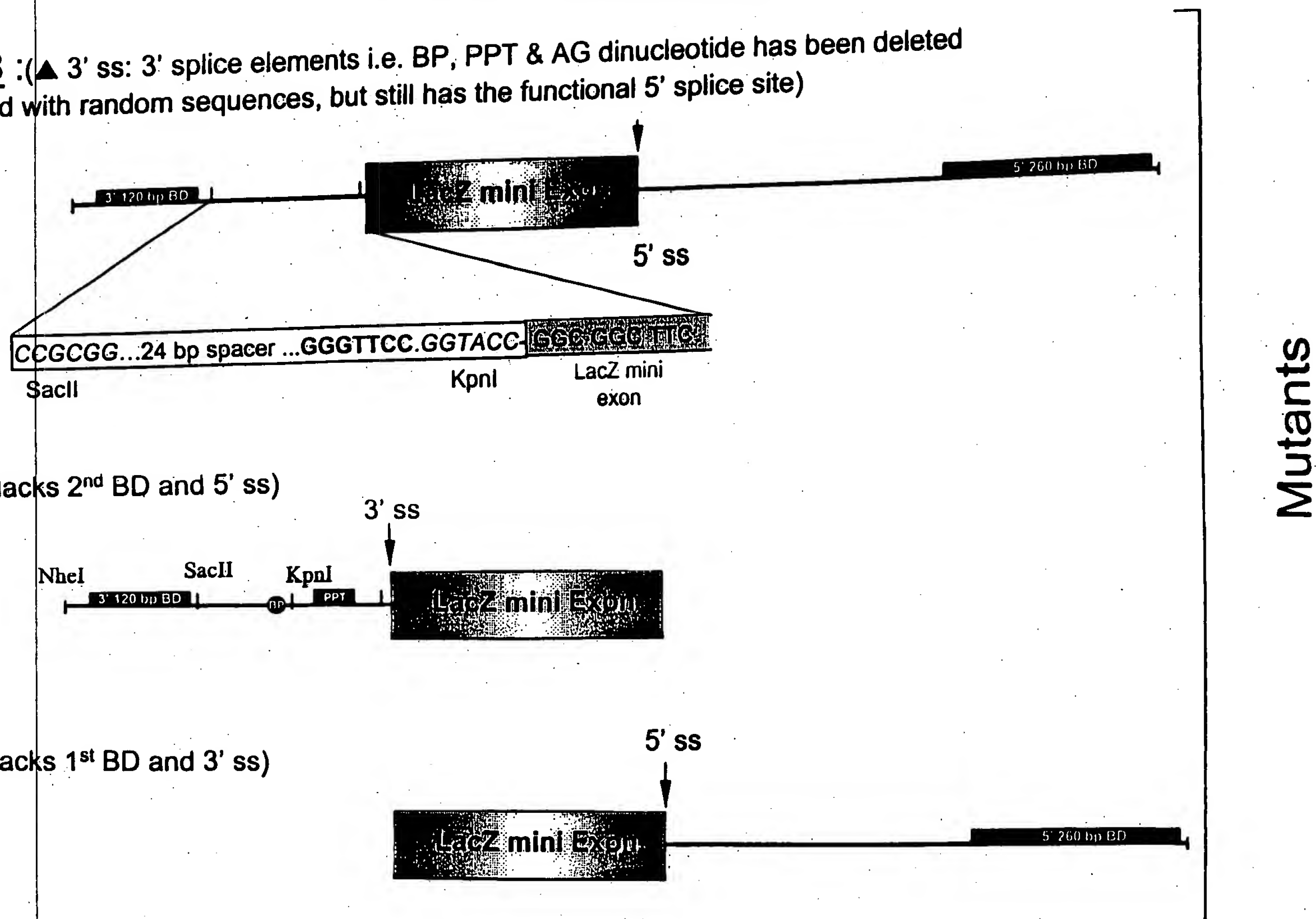
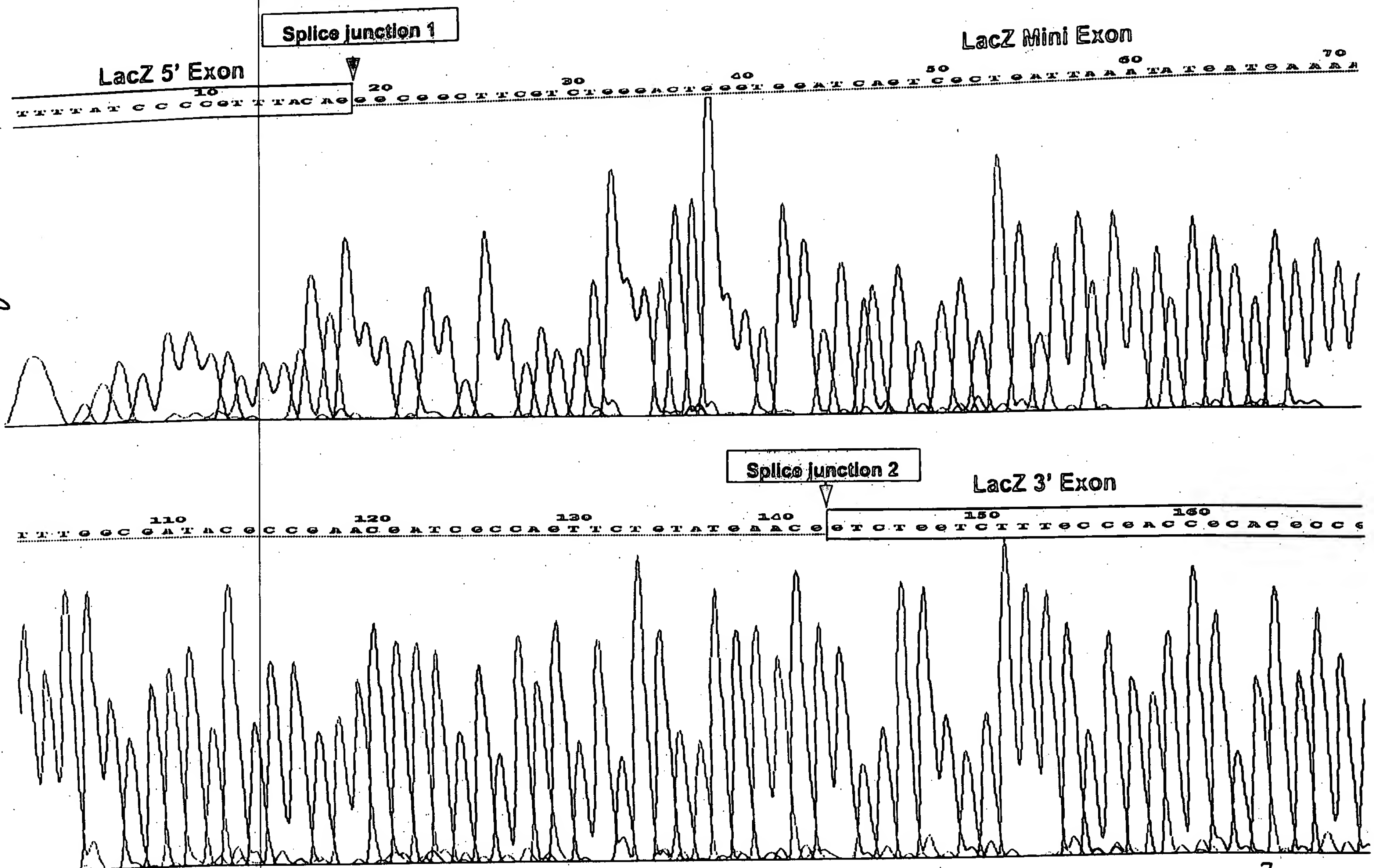


Figure 22

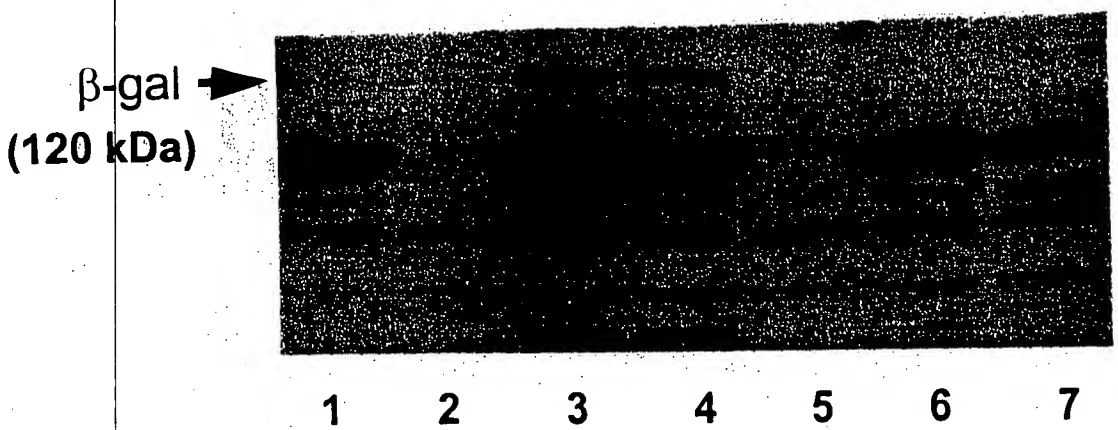
Accuracy of Double *Trans*-splicing Reaction



7

Figure 23

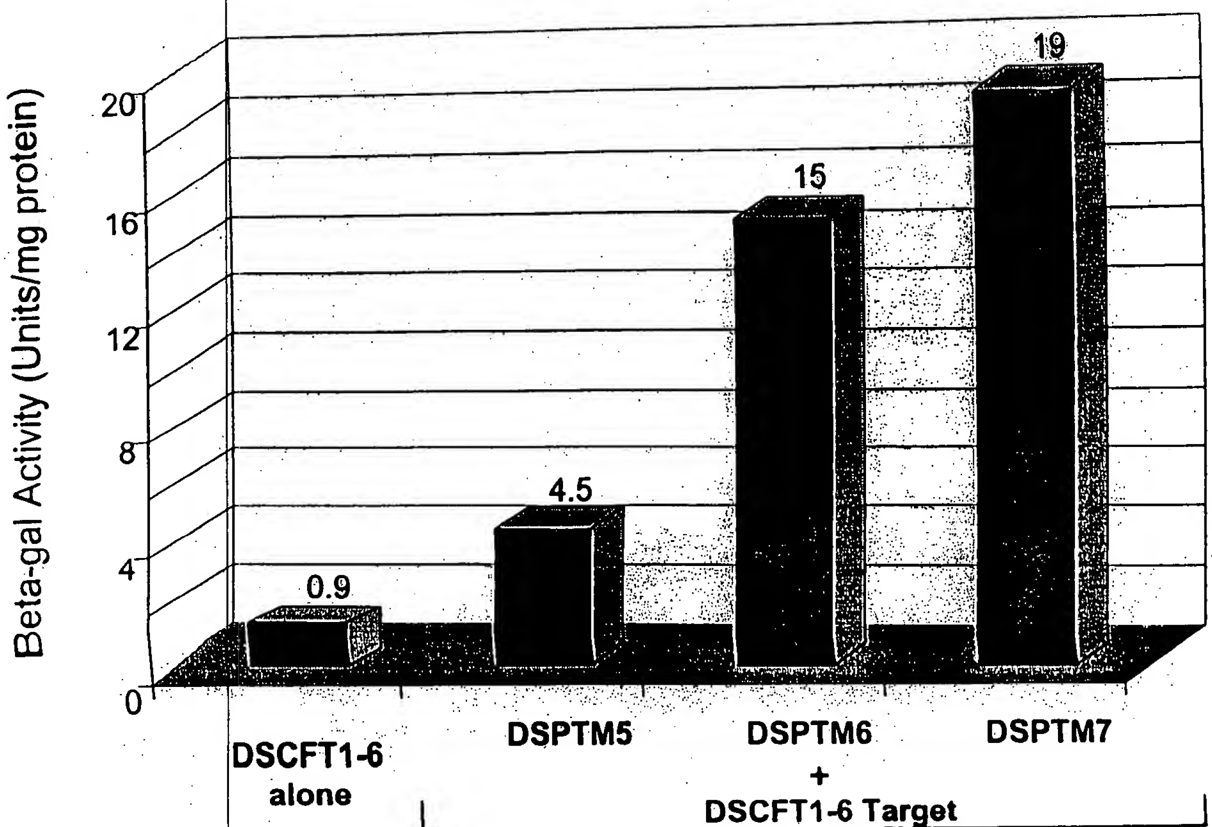
Double *Trans*-splicing Produces Full-length Protein



Lane 1: DSCFT1.6 Target alone	25 μg
Lane 2: DSPTM7	25 μg
Lane 3 Target + PTM #6	25 μg
Lane 4: Target + PTM #9	25 μg
Lane 5: Delta 3' splice mutant alone	25 μg
Lane 6: Target + Delta 3' ss	25 μg
Lane 7: Target+PTM29+30 (mutants)	25 μg

Figure 24

Restoration of β-Gal Function by Double *Trans*-splicing

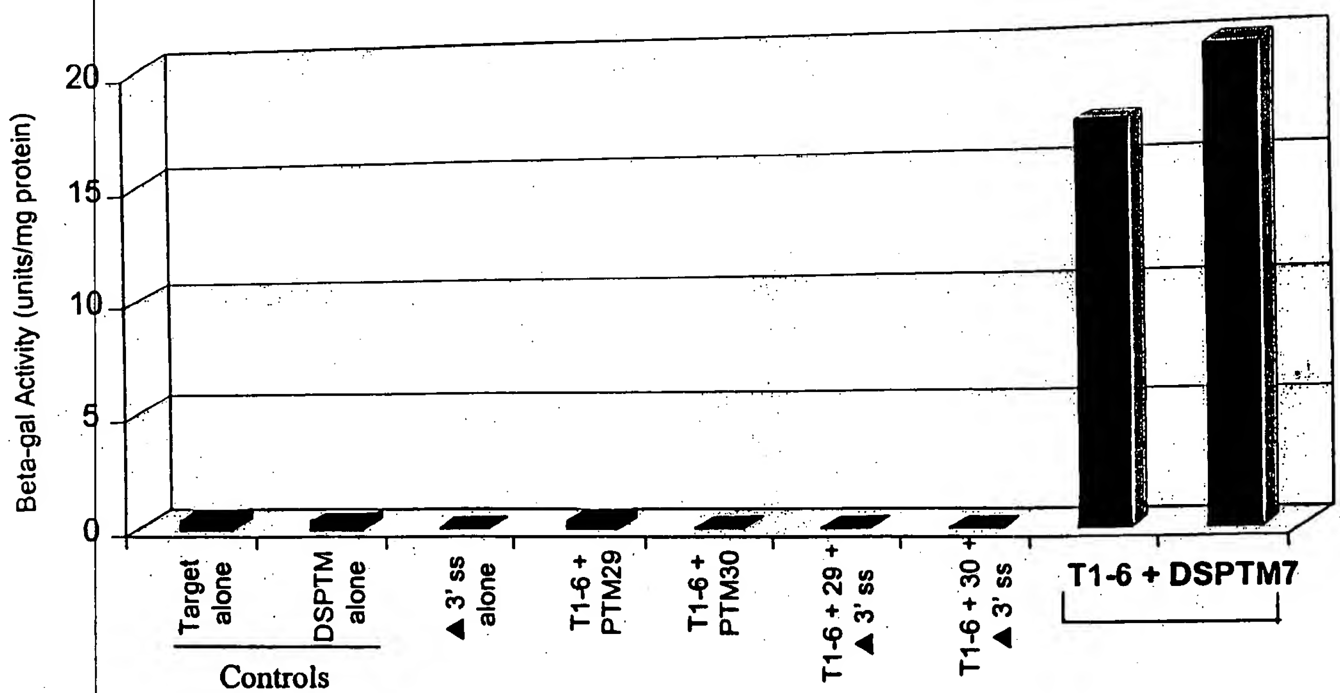


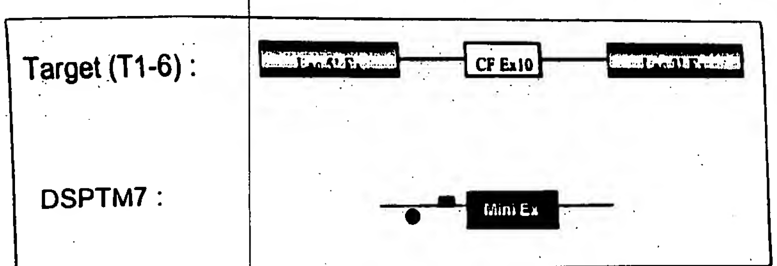
Beta-gal Activity above the Background level

DSPTM5: 5 fold
DSPTM6: 17 fold
DSPTM7: 21 fold

Figure 25

Restoration of β-gal activity is due to double RNA *trans*-splicing events





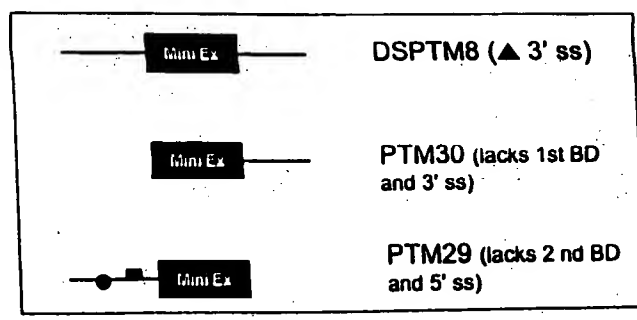
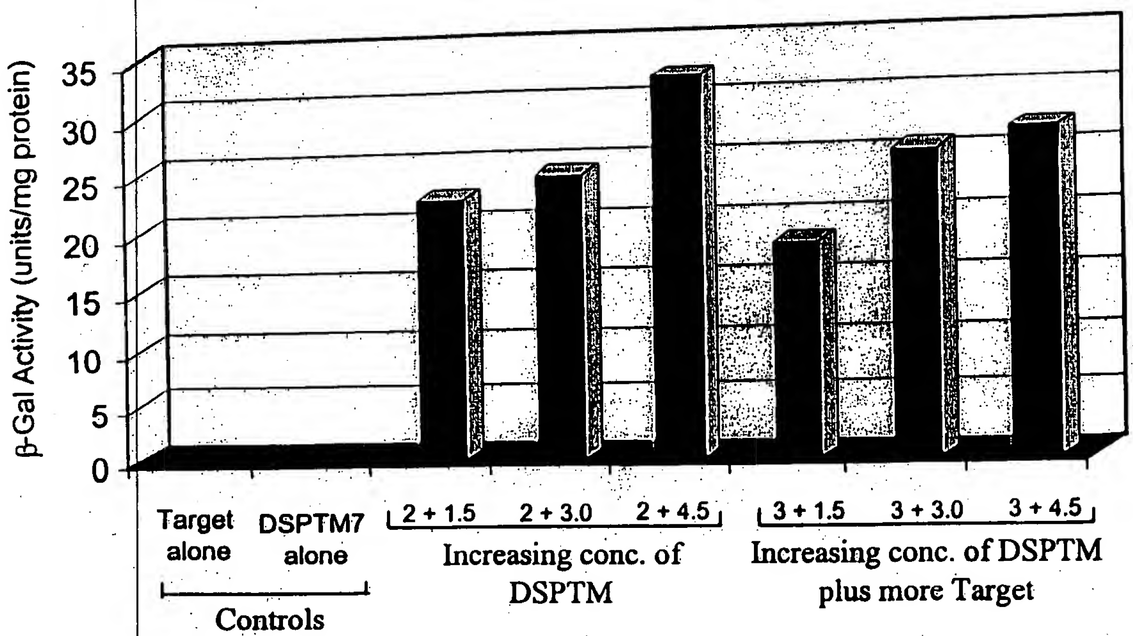


Figure 26

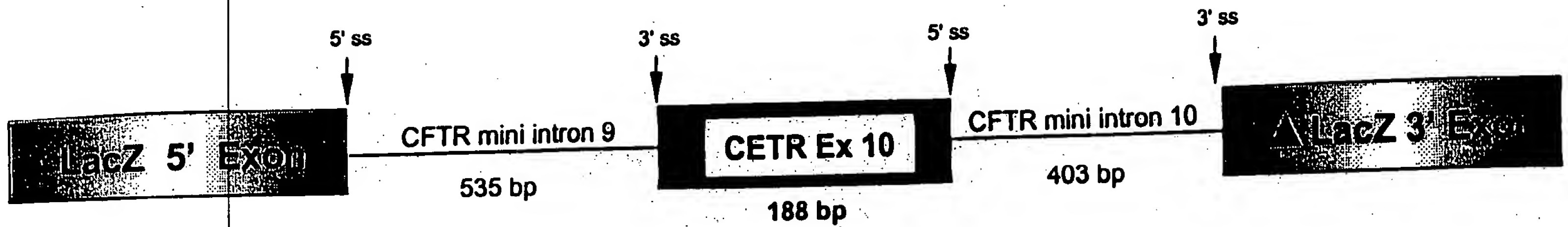
Double *Trans*-splicing: Titration of Target & PTM



The current level of beta-gal activity due to double *trans*-splicing is ~ 1-1.5% of the best single splice model (3' exon replacement)

Figure 27

DSCFT1-6 (Specific Target):



DSHCGT1 (Non-specific Target):

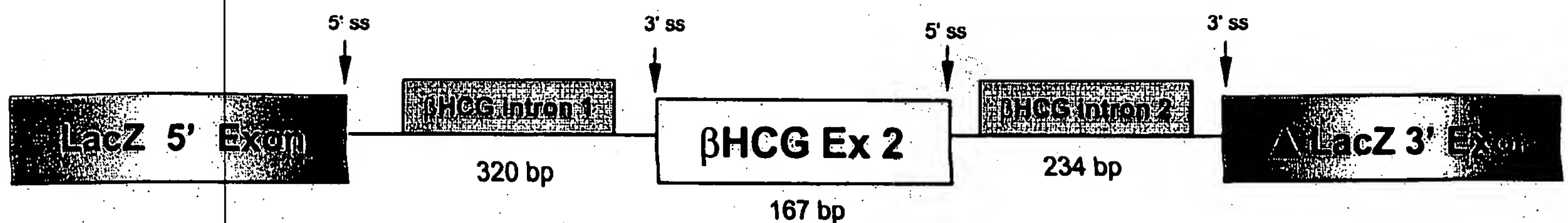


Figure 28

Sheet 35 of 66

Specificity of double *trans*-splicing Reaction

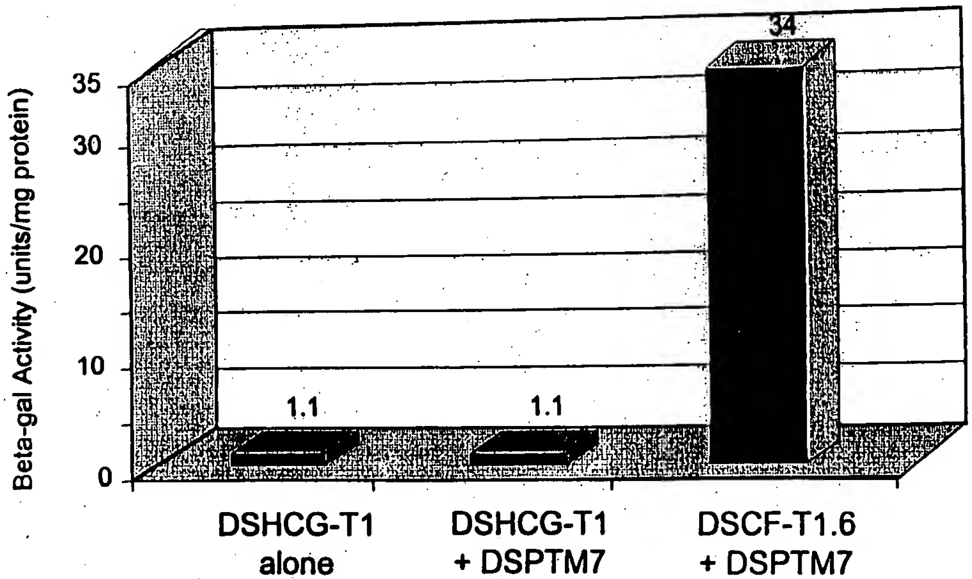


Figure 29

Replacement of a Single Internal Exon

Schematic diagram of a PTM binding to a CFTR ΔF508 target

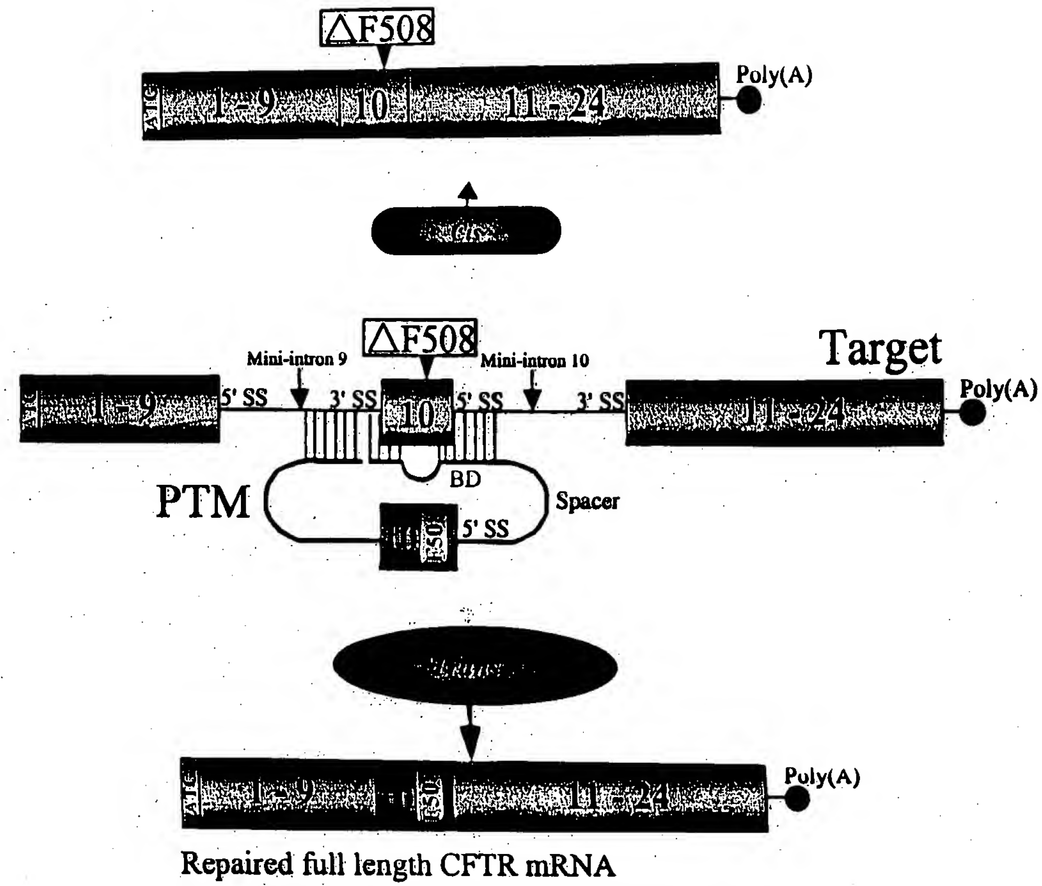


Figure 30

INTRONN

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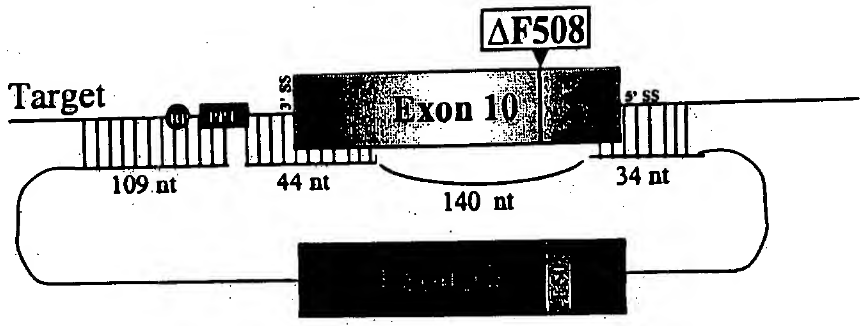
Sheet 38 of 66

PTM with a long binding domain masking
two splice sites and part of exon 10
in a mini-gene target.



PTM

ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

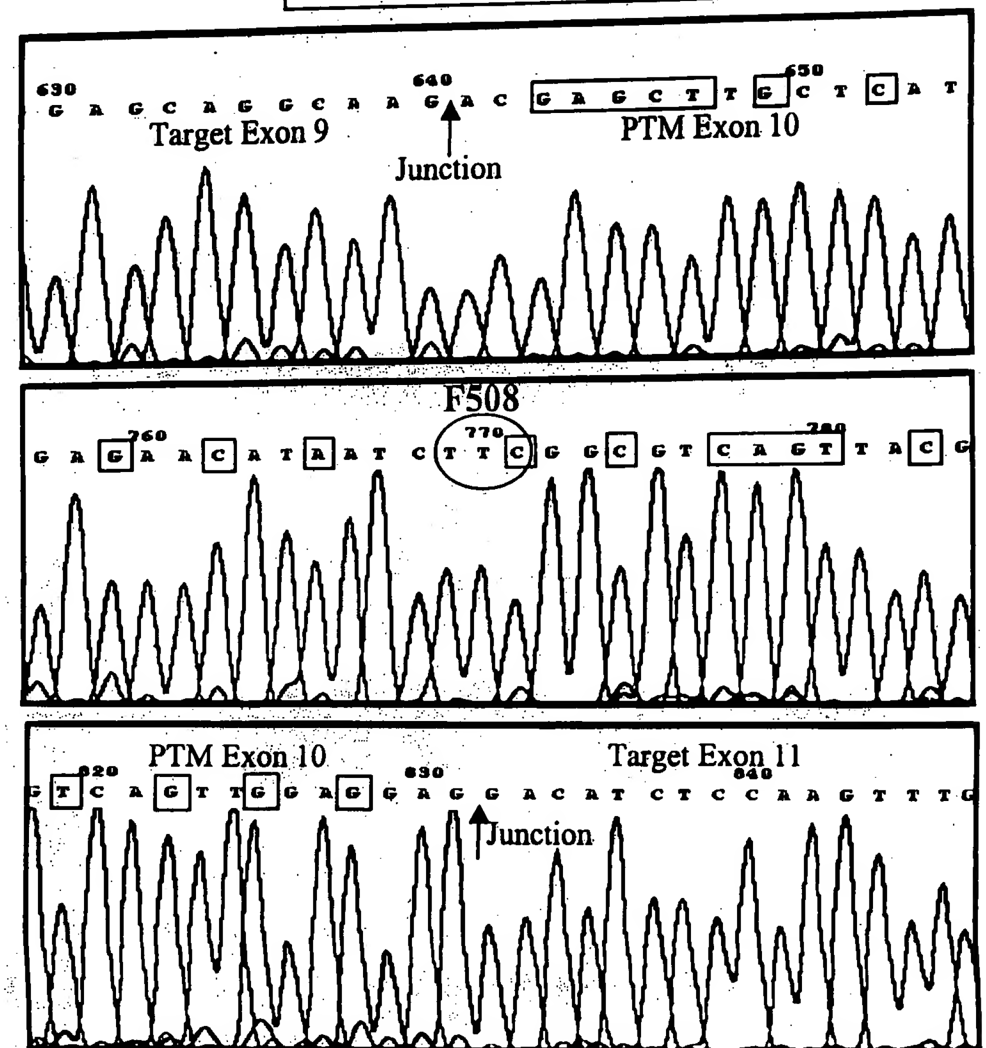
MCU in exon 10 of PTM

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

Figure 31

INTRONN

Sequence of a double
trans-spliced product



□ = MCU in
PTM exon 10

Figure 32

Sheet 39 of 66

CFTR Repair: 5' Exon Replacement

Schematic diagram of a PTM binding to the splice site of intron 10 of a mini-gene target

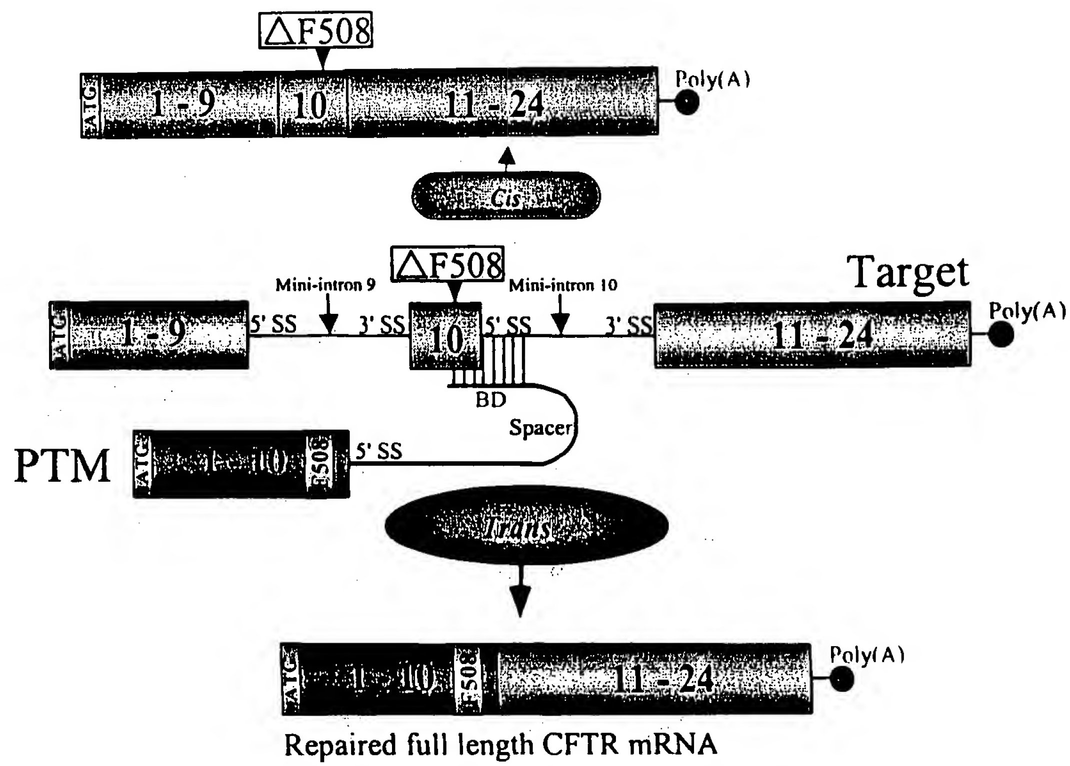


Figure 33

Sheet 40 of 66

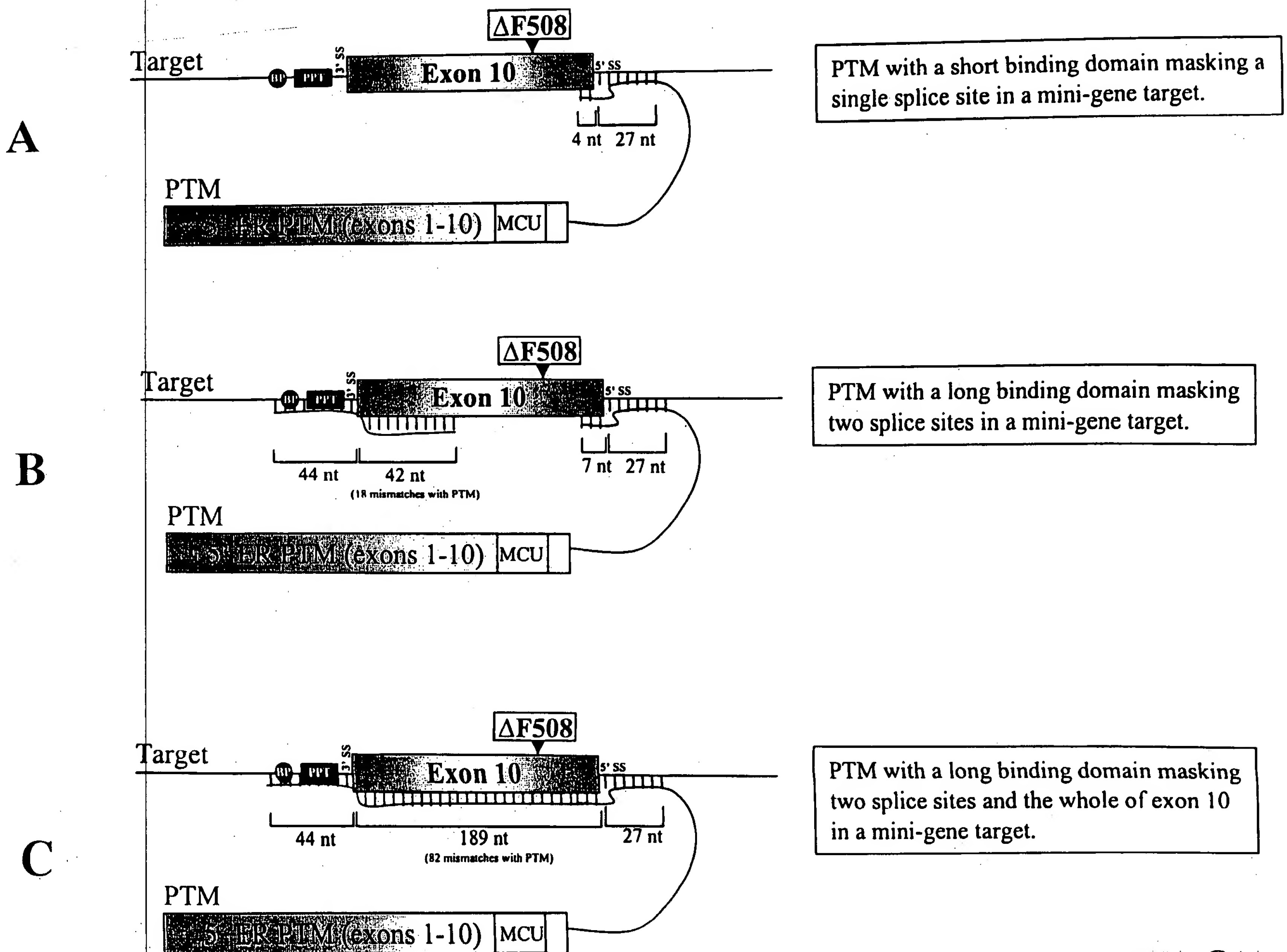
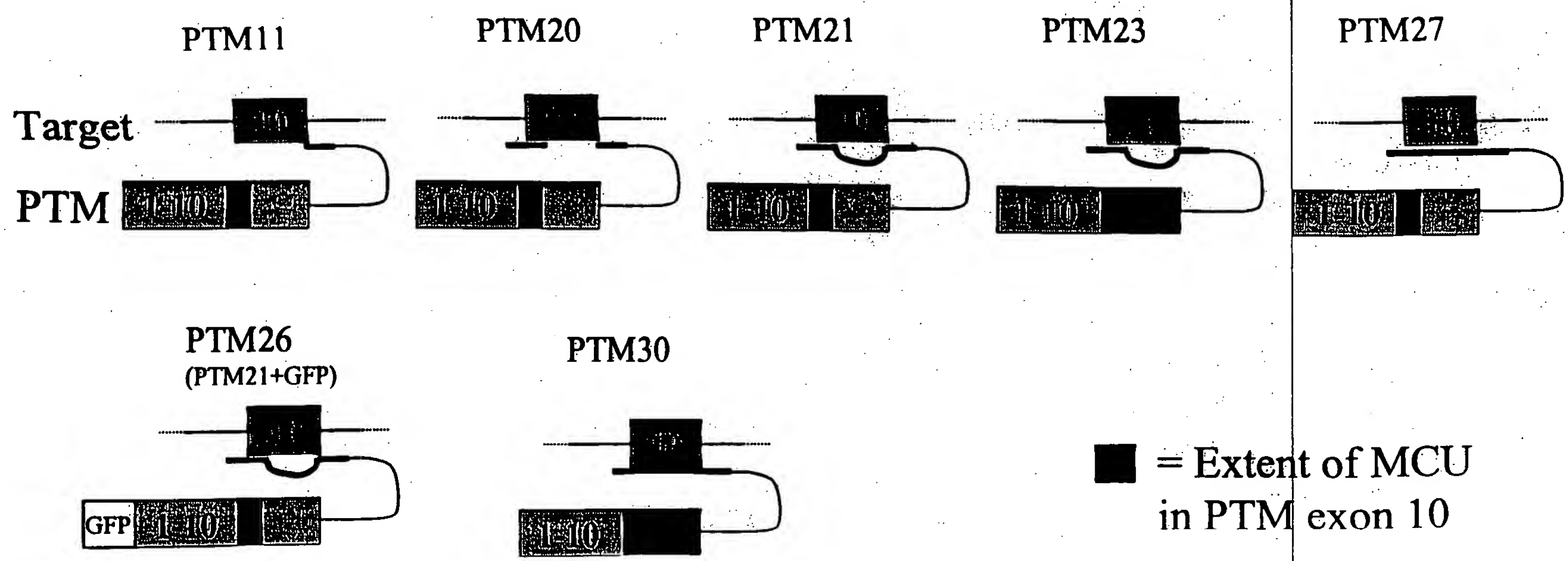

A
B
C
Target
PTM
ΔF508
Exon 10
PPT
BP
3' SS
5' SS
4 nt
27 nt
7 nt
44 nt
42 nt
(18 mismatches with PTM)
189 nt
(82 mismatches with PTM)
5' ER PTM (exons 1-10)
MCU
PTM with a short binding domain masking a single splice site in a mini-gene target.
PTM with a long binding domain masking two splice sites in a mini-gene target.
PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

Figure 34



<u>MCU in exon 10 of PTM</u>

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

Figure 35

09838858 .042001

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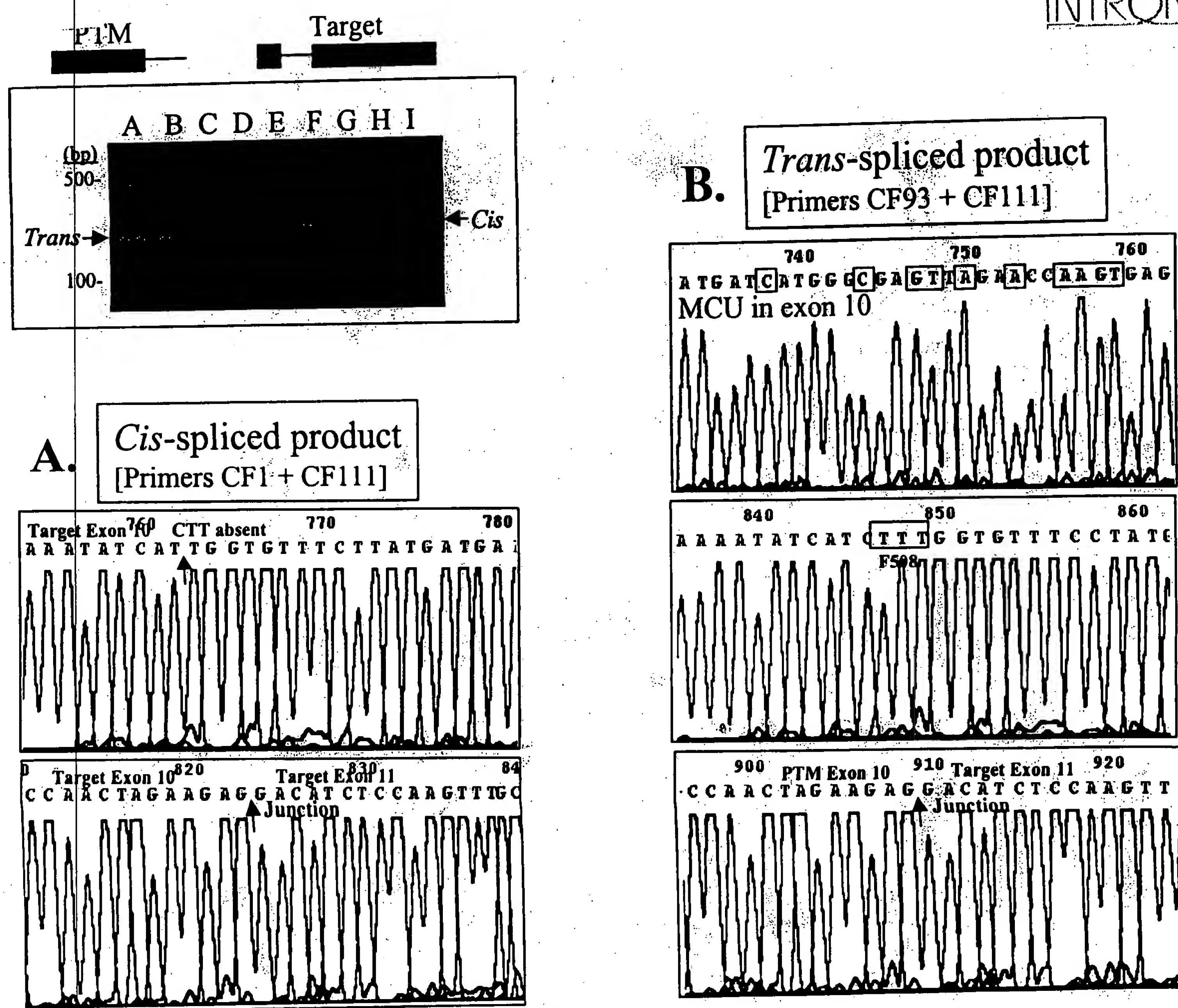
INTRONN



5

Figure 36

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A

lacZCF9m

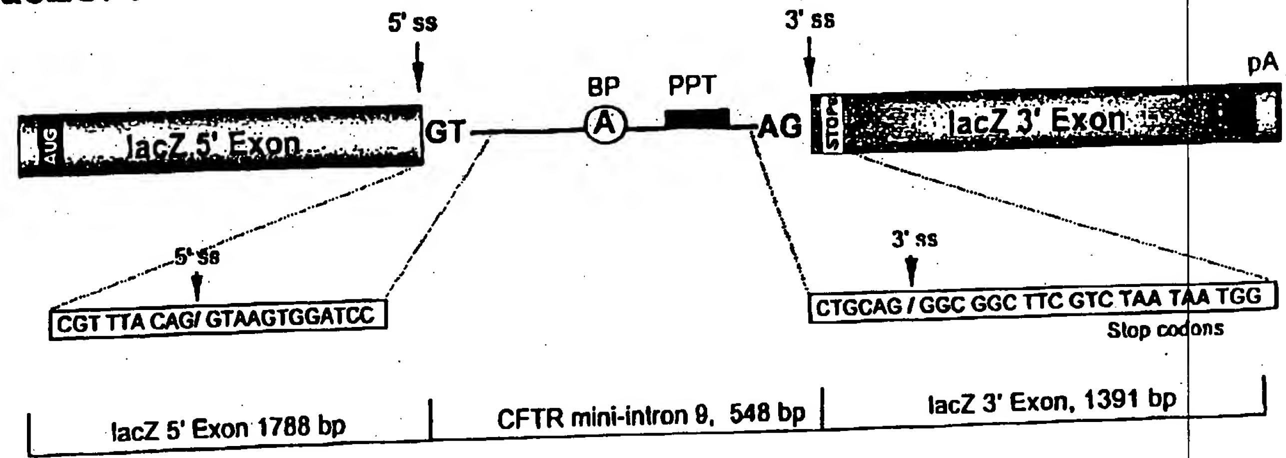

Figure 37 A

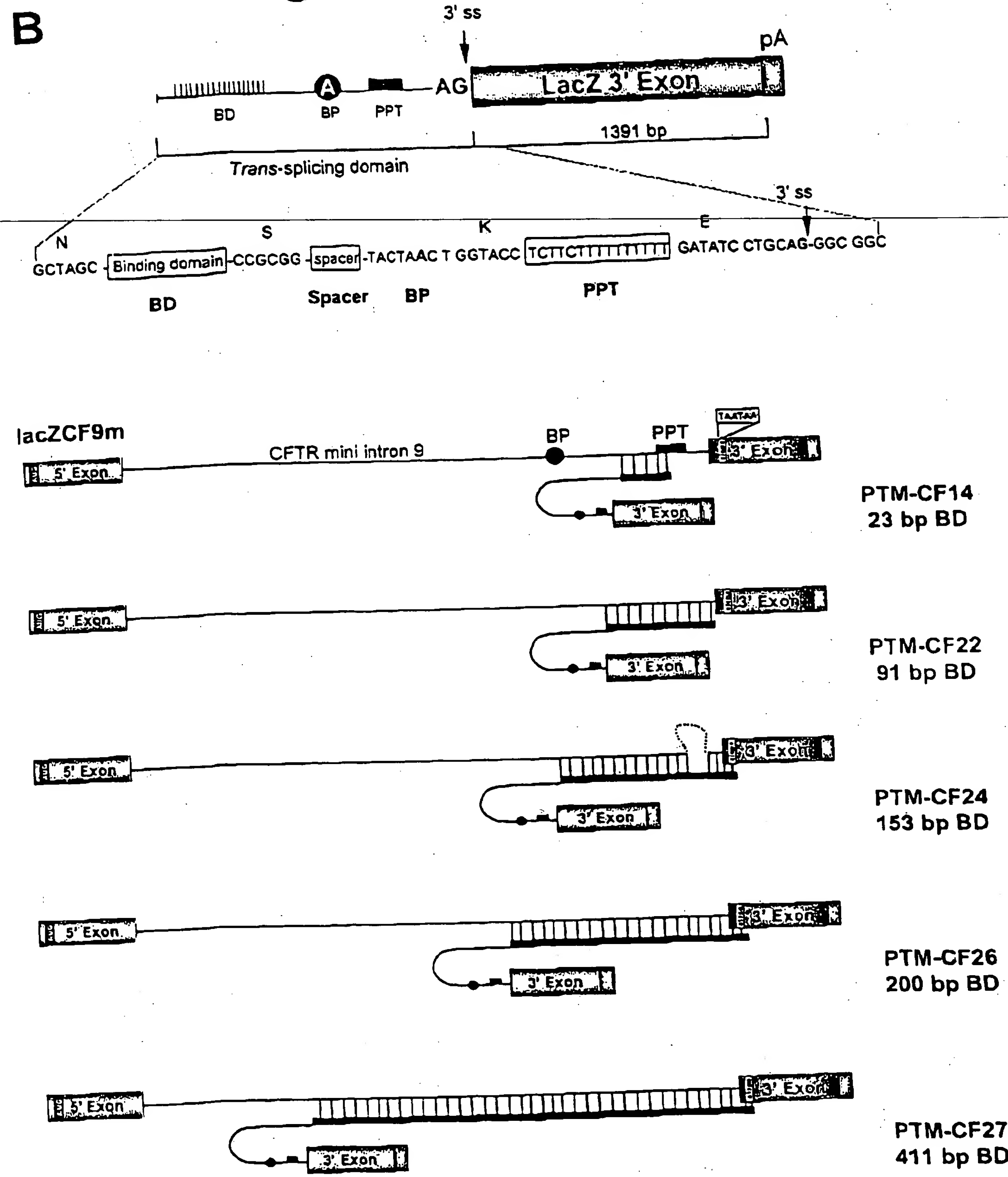
B
3' ss
pA
AG
LacZ 3' Exon
BD
BP
PPT
1391 bp
Trans-splicing domain
3' ss
N
S
K
E
GCTAGC
Binding domain
CCGCGG
spacer
TACTAAC T GGTACC
TCTTCTTTTTTTTTTT
GATATC CTGCAG-GGC GGC
BD
Spacer
BP
PPT
lacZCF9m
CFTR mini intron 9
BP
PPT
5' Exon
3' Exon
PTM-CF14
23 bp BD
PTM-CF22
91 bp BD
PTM-CF24
153 bp BD
PTM-CF26
200 bp BD
PTM-CF27
411 bp BD

Figure 37B

C

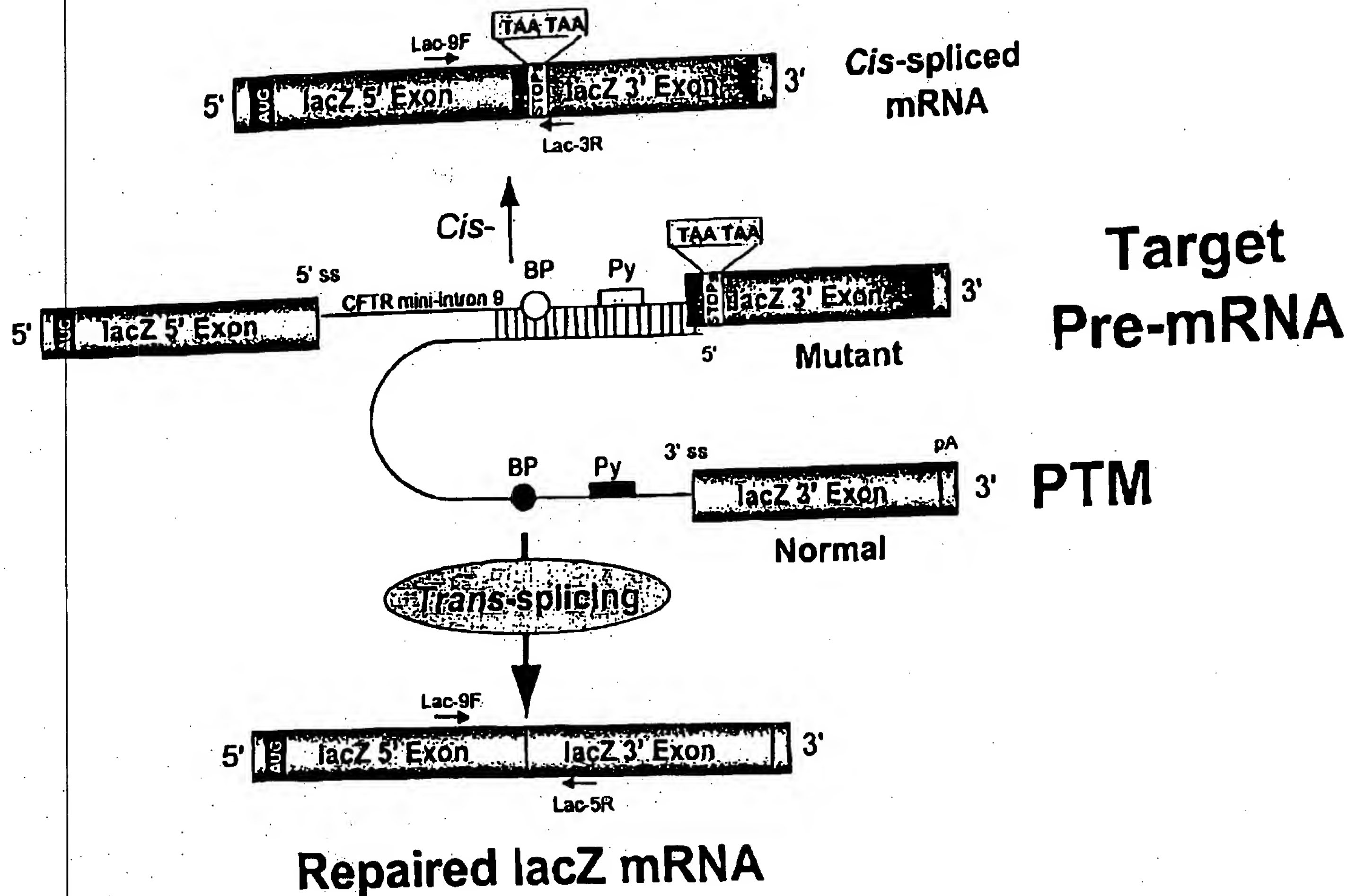

Figure 37C

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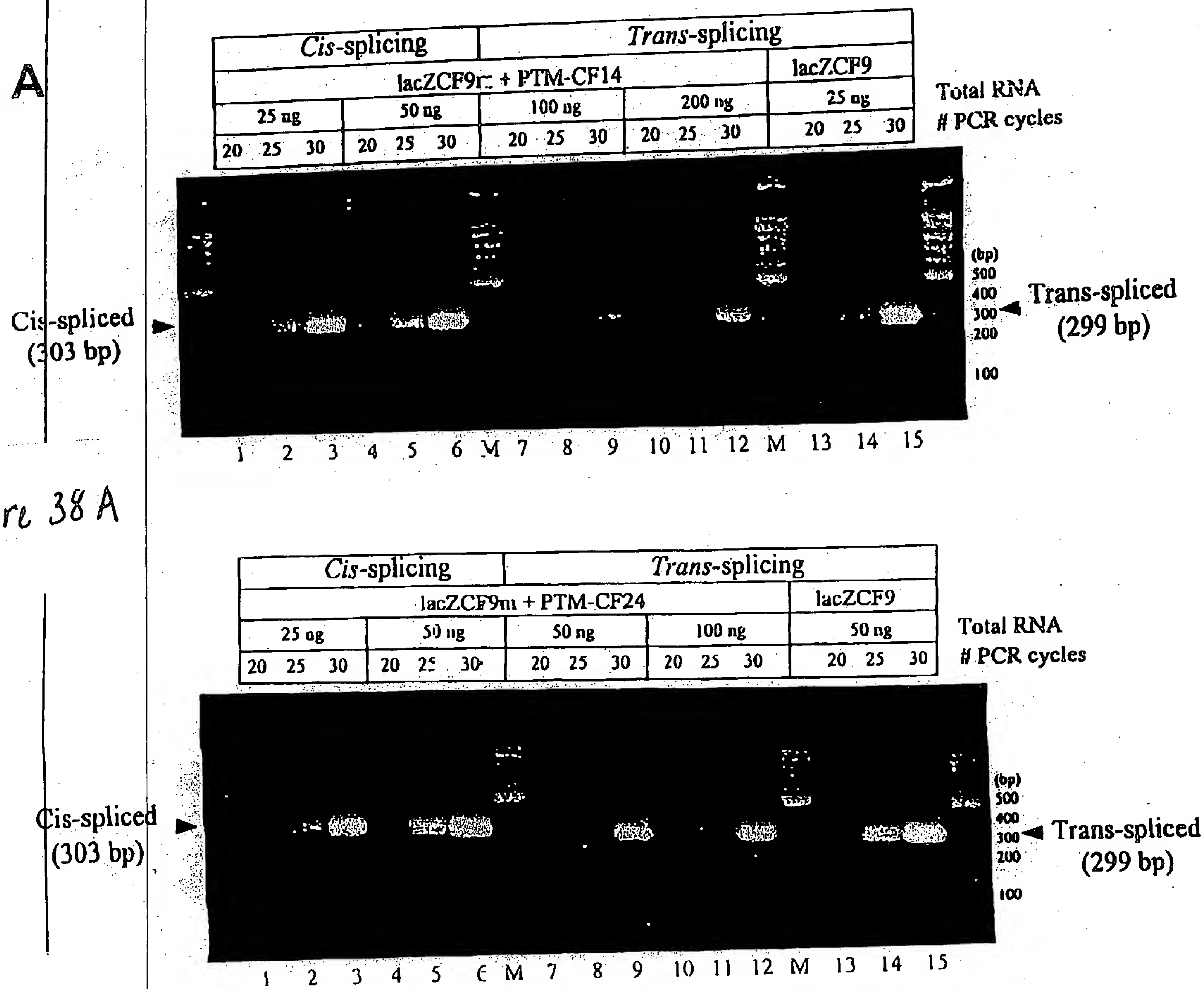
A
Cis-splicing
Trans-splicing
lacZCF9m + PTM-CF14
lacZCF9
25 ng
50 ng
100 ng
200 ng
25 ng
20 25 30
Total RNA
PCR cycles
(bp)
500
400
300
200
100
Cis-spliced (303 bp)
Trans-spliced (299 bp)
1 2 3 4 5 6 M 7 8 9 10 11 12 M 13 14 15
Cis-splicing
Trans-splicing
lacZCF9m + PTM-CF24
lacZCF9
25 ng
50 ng
50 ng
100 ng
50 ng
20 25 30
Total RNA
PCR cycles
Cis-spliced (303 bp)
Trans-spliced (299 bp)
1 2 3 4 5 6 M 7 8 9 10 11 12 M 13 14 15

Figure 38 A

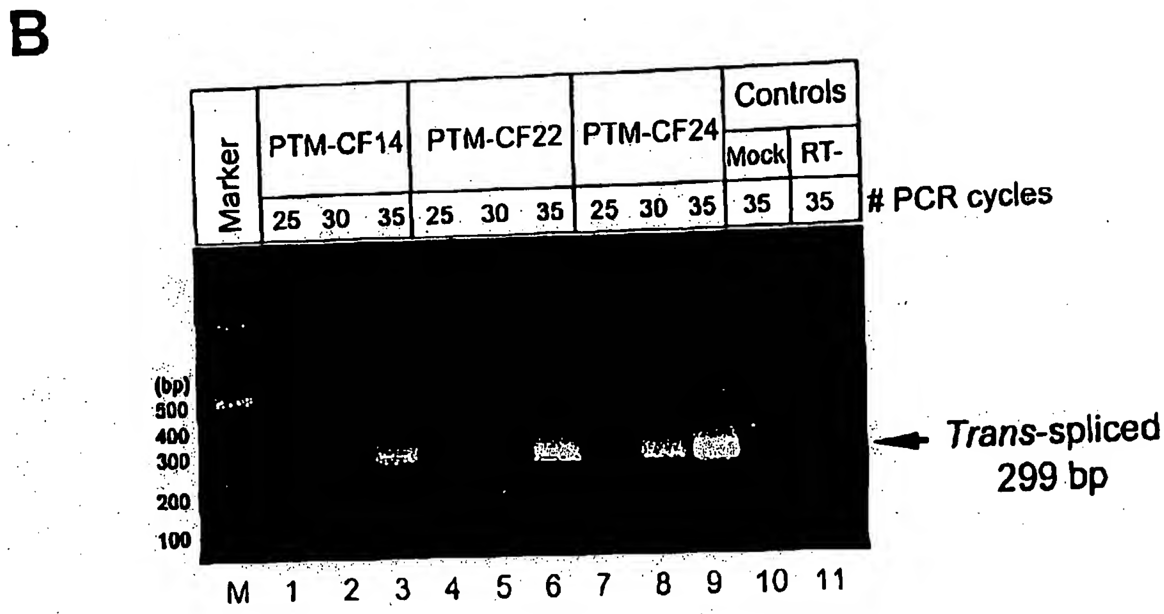
B
Marker
PTM-CF14
PTM-CF22
PTM-CF24
Controls
Mock
RT-
25 30 35 25 30 35 25 30 35 35 35
PCR cycles
(bp)
500
400
300
200
100
Trans-spliced
299 bp
M 1 2 3 4 5 6 7 8 9 10 11

Figure 38B

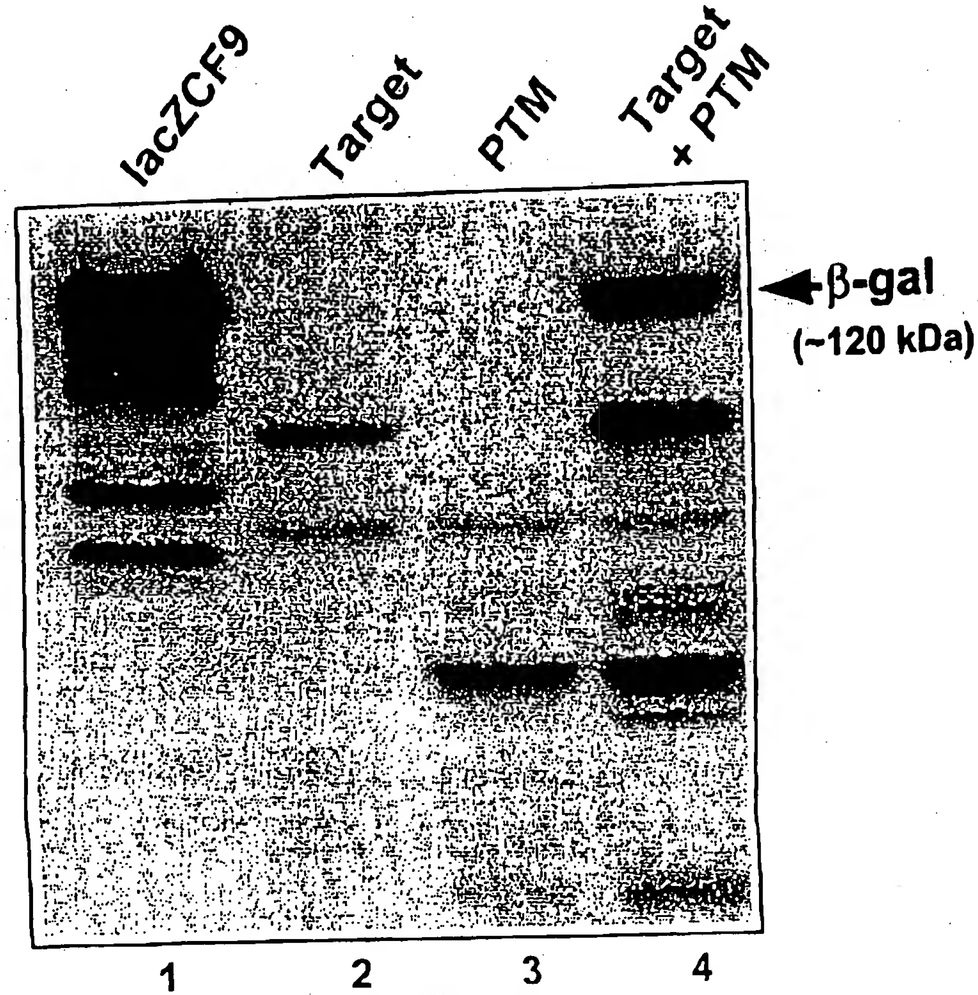

Figure 39

A

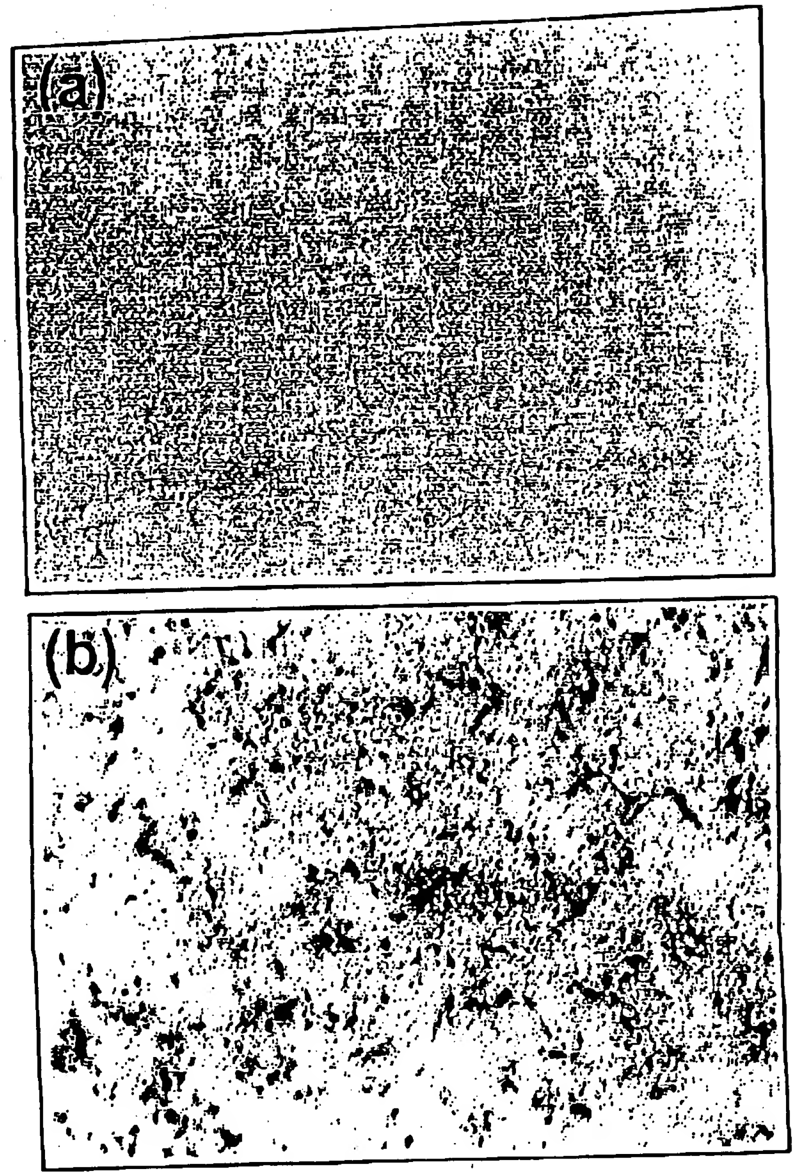

Figure 40A

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B

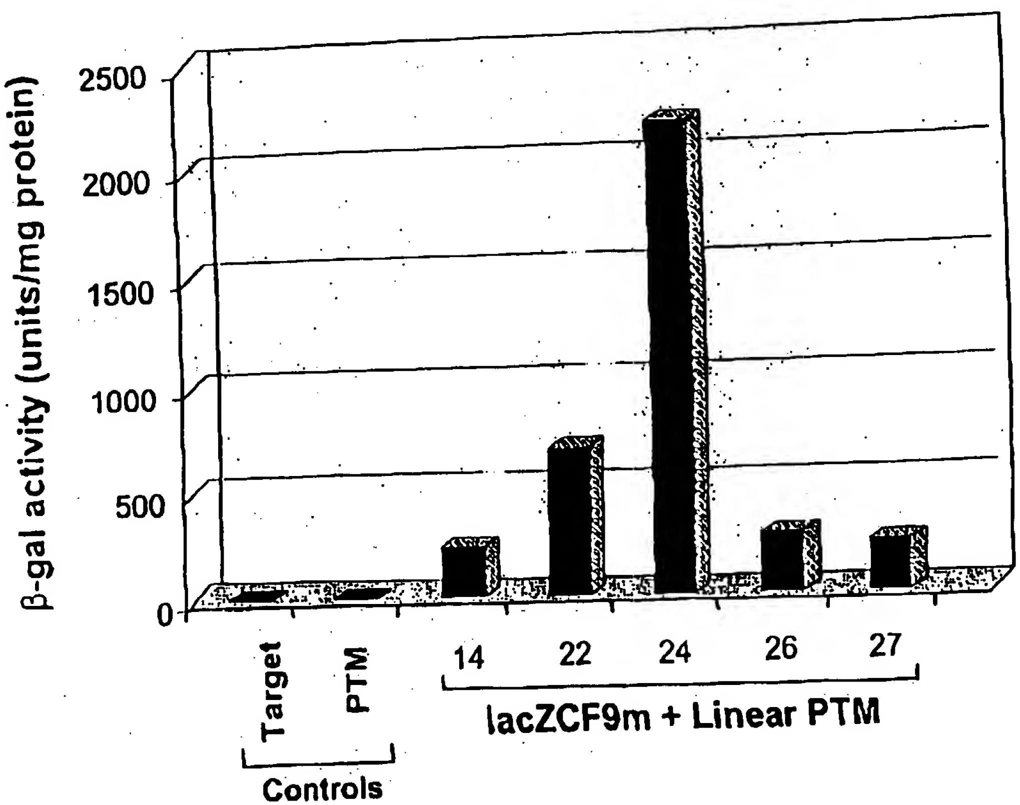

β-gal activity (units/mg protein)
2500
2000
1500
1000
500
0
Target
PTM
Controls
14
22
24
26
27
lacZCF9m + Linear PTM

Figure 40B

C

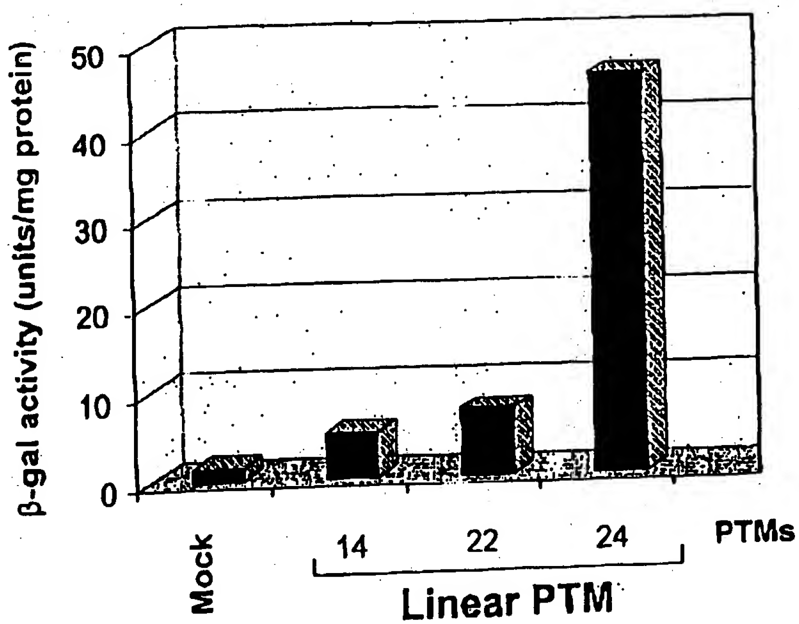

β-gal activity (units/mg protein)
50
40
30
20
10
0
Mock
14
22
24
PTMs
Linear PTM

Figure 40C

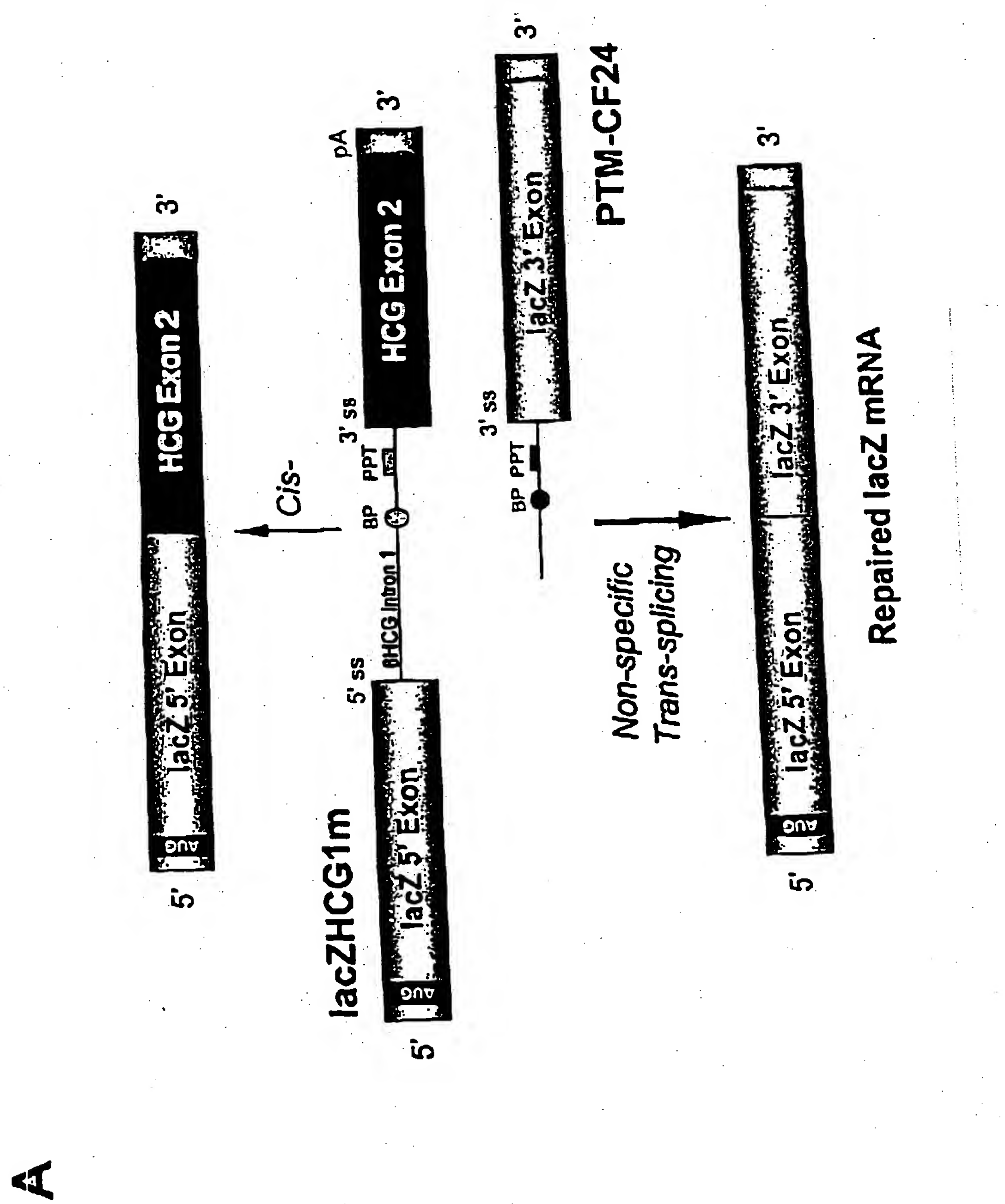

A
lacZ 5' Exon
HCG Exon 2
AUG
5'
3'
Cis-
lacZHCG1m
5' ss
βHCG Intron 1
BP
PPT
3' ss
pA
PTM-CF24
lacZ 3' Exon
Non-specific
Trans-splicing
Repaired lacZ mRNA

Figure 41A

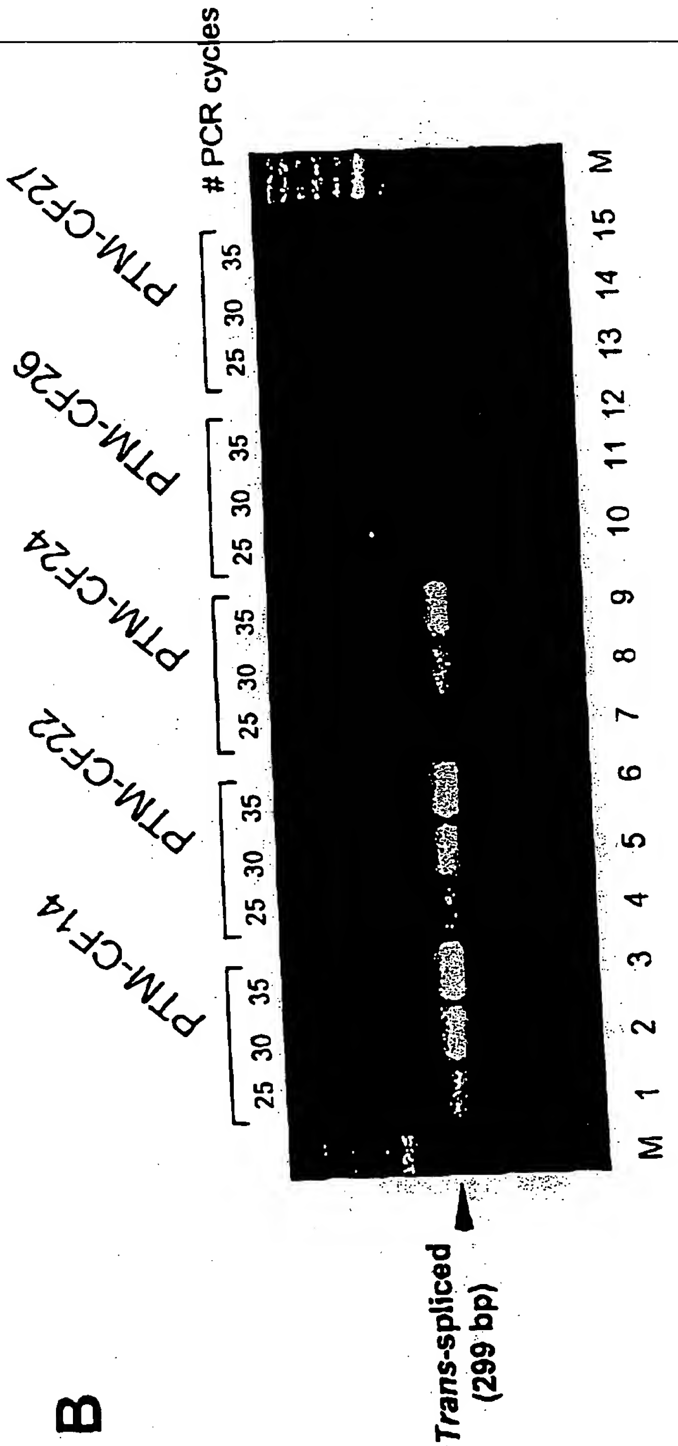

B
PTM-CF14
PTM-CF22
PTM-CF24
PTM-CF26
PTM-CF27
PCR cycles
25 30 35
25 30 35
25 30 35
25 30 35
25 30 35
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
Trans-spliced
(299 bp)

Figure 4B

C

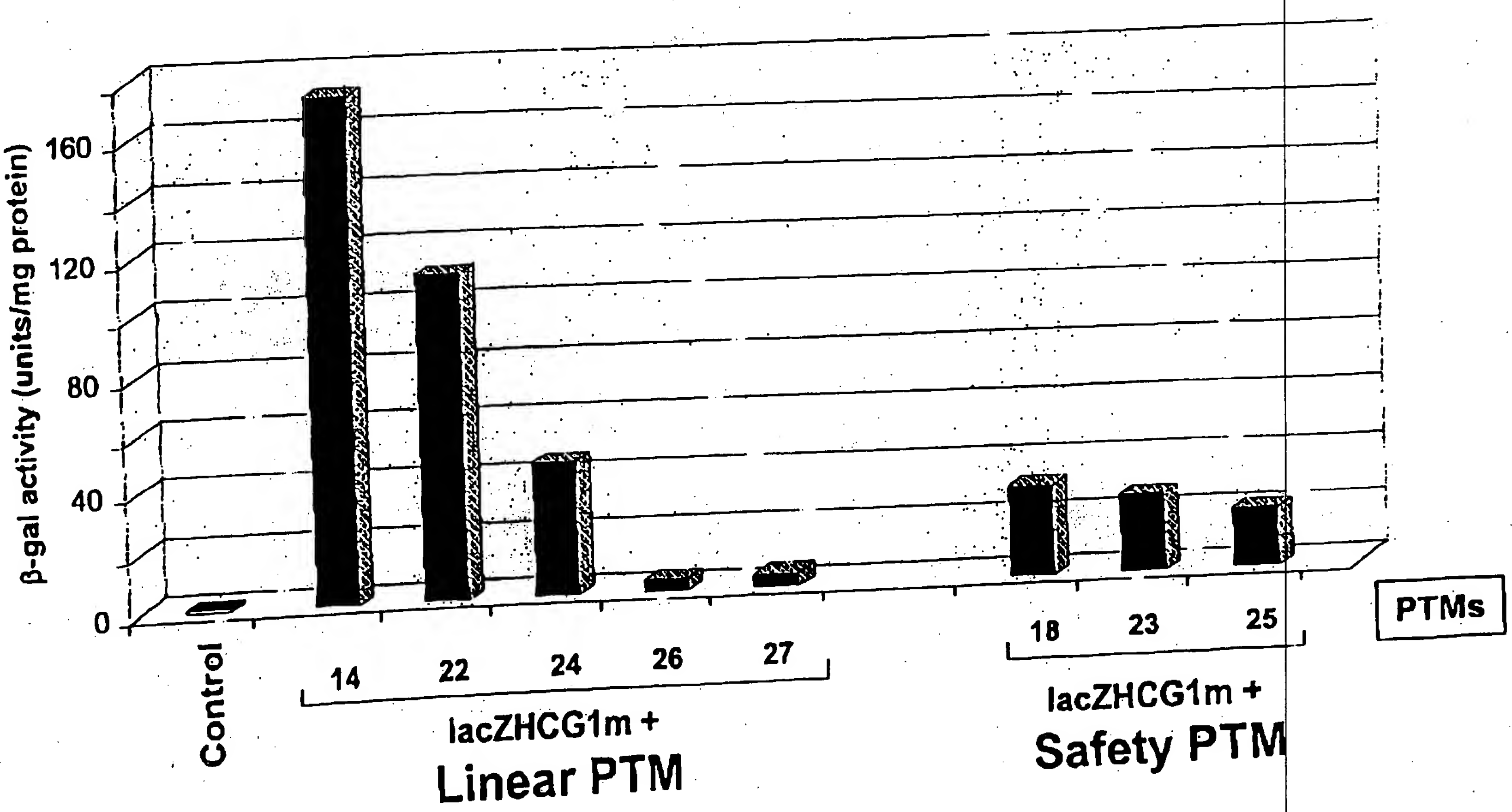

β-gal activity (units/mg protein)
0
40
80
120
160
Control
14
22
24
26
27
lacZHCG1m +
Linear PTM
18
23
25
lacZHCG1m +
Safety PTM
PTMs

Figure 41C

Exons 1-10

ATGCAGAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTTTTCAGCTGGACCAGACCAATTTTGAGGAAAG
GATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCCTTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT
GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTCTGG
AGATTTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCTCTCTTACTGGGAAGAATCA
TAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTAT
TGTGAGGACACTGCTCCTACACCCAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGT
TTGATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACTTGTTAGTCTCCTTT
CCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCACATTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCT
CATGGGGCTAATCTGGGAGTTGTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTTTTCAG
GCTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACA
AACAGAACTGAAACTGACTCGGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCCTCCGGAAAATATTCACCACCATCTCATTCT
GCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAA
CAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
AATGTAACAGCCTTCTGGGAGGAGGGATTTGGGGAATTATTTGAGAAAGCAAAACAAAACAATAACAATAGAAAAACTT
CTAATGGTGATGACAGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTTCAAGAT
AGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGCTCATGATGATCATGGGCGAG
TTAGAACCAAGTGAAGGCAAGATCAAACATTCCGGCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTA
CCATCAAGGAGAACATAATCTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGA
GGAG

Trans-splicing domain

GTAAGATATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACGCTAAGATCCACCGG
TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTTGAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATCATTG
GAAACACCAATGATATTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGT
GCTTAATTTTACCCTCTGAATTCTCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATT
ATTAACTCATTATCAAATCACGCT

Figure 42

153 bp PTM24 Binding Domain:

Nhe I   153 bp BD underlined

GCTAGC-AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

TTCTTATTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II

AC-**CCGCGG**

Figure 43A

Trans-splicing domain

AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTA
TTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGCACCCGC
GGAACATTATTATAACGTTGCTCGAATACTAACTGGTACCTCTTCTTTTTTTTTTGATATCCTGCAG

Exons 10-24

ACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGAAGAATTTCATTCT
GTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGATA
CAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAA
GGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATT
TATTAGACTCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAAACTGATGGC
TAACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAATATTAATTTTGCATGAAGGT
AGCAGCTATTTTTATGGGACATTTTCAGAACTCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATT
CTTTCGACCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCATTAGAAGGAGATGC
TCCTGTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGGAAAAAAGGAAGAATTCTATT
CTCAATCCAATCAACTCTATACGAAAATTTTCCATTGTGCAAAAGACTCCCTTACAAATGAATGGCATCGAAGAGGATT
CTGATGAGCCTTTAGAGAGAAGGCTGTCCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACTGCCTCGCATCAGCGT
GATCAGCACTGGCCCCACGCTTCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCAGTTAACCAAGGT
CAGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGCAAACTTGACTGAACTGGATA
TATATTCAAGAAGGTTATCTCAAGAAACTGGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTT
TTTTGATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTTA
ATTTTTGTGCTAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCTTCTTTGGTTGTGCTGTGGCTCCTTGGAA
ACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTC
GTATTATGTGTTTTACATTTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCACTGGTG
CATACTCTAATCACAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTTCTTCAAGCACCTATGTCAACCCTCA
ACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCAT
ATTTGACTTCATCCAGTTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTTGTT
GCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCCAAACCTCACAGCAACTCAAACAACTGG
AATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTTACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACG
GCAGCCTTACTTTGAAACTCTGTTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTACCTGTCAACACTG
CGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCATCTTCTTCATTGCTGTTACCTTCATTTCCATTTTAACAACAG
GAGAAGGAGAAGGAAGAGTTGGTATTATCCTGACTTTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTC
CAGCATAGATGTGGATAGCTTGATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCT
ACCAAGTCAACCAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATG
ACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACAGCAAAATACACAGAAGGTGGAAATGCCATATTAGA
GAACATTTCCTTCTCAATAAGTCCTGGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTA
TCAGCTTTTTTGAGACTACTGAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAAC
AGTGGAGGAAAGCCTTTGGAGTGATACCACAGAAAGTATTTATTTTTTCTGGAACATTTAGAAAAAACTTGGATCCCTA
TGAACAGTGGAGTGATCAAGAAATATGGAAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTTTCCTGGG
AAGCTTGACTTTGTCCTTGTGGATGGGGGCTGTGTCCTAAGCCATGGCCACAAGCAGTTGATGTGCTTGGCTAGATCTG
TTCTCAGTAAGGCGAAGATCTTGCTGCTTGATGAACCCAGTGCTCATTTGGATCCAGTAACATACCAAATAATTAGAAG
AACTCTAAAACAAGCATTTGCTGATTGCACAGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAA
TTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAACTGCTGAACGAGAGGAGCCTCTTCCGGC
AAGCCATCAGCCCCTCCGACAGGGTGAAGCTCTTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCCCCAGATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTCATCATCATCATCATCATTAG

Figure 43B

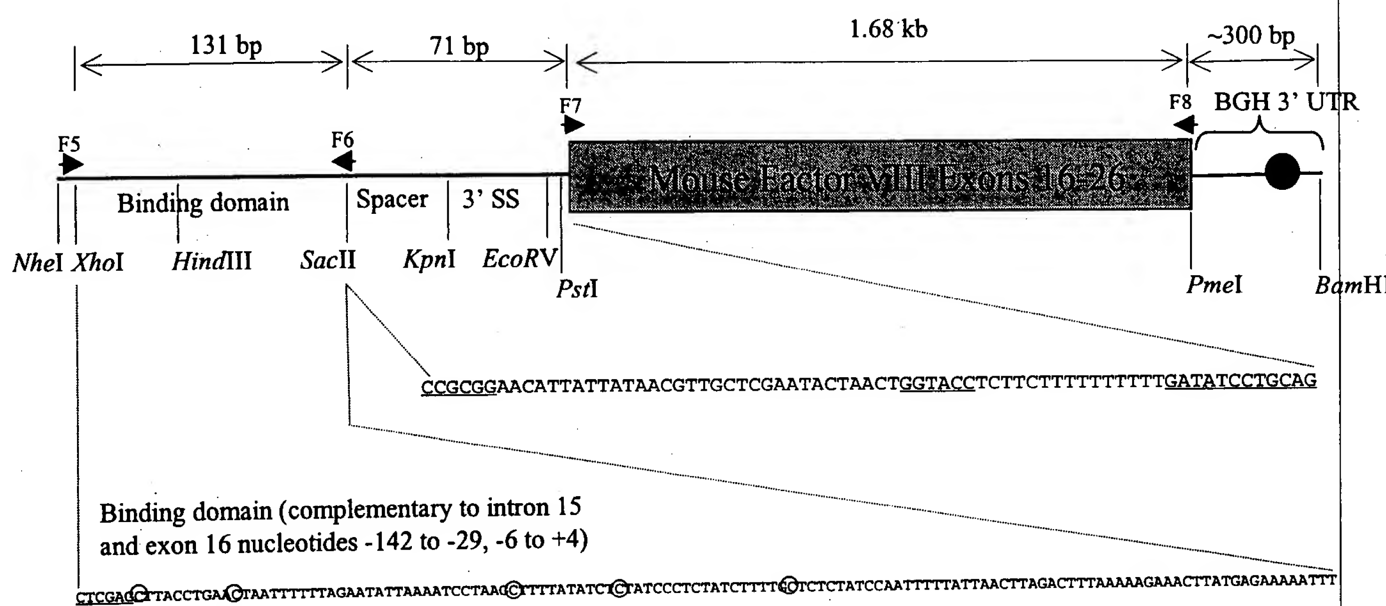

131 bp
71 bp
1.68 kb
~300 bp
F5
F6
F7
F8
BGH 3' UTR
GT
Binding domain
Spacer
3' SS
Mouse Factor VIII Exons 16-26
NheI
XhoI
HindIII
SacII
KpnI
EcoRV
PstI
PmeI
BamHI
CCGCGGAACATTATTATAACGTTGCTCGAATACTAACTGGTACCTCTTCTTTTTTTTTGATATCCTGCAG
Binding domain (complementary to intron 15
and exon 16 nucleotides -142 to -29, -6 to +4)
CTCGAGCTTACCTGAACTAATTTTTTAGAATATTAAAATCCTAAGCTTTTATATCTCTATCCCTCTATCTTTTGCTCTCTATCCAATTTTTATTAACTTAGACTTTAAAAAGAAACTTATGAGAAAAATTT

Figure 44 A

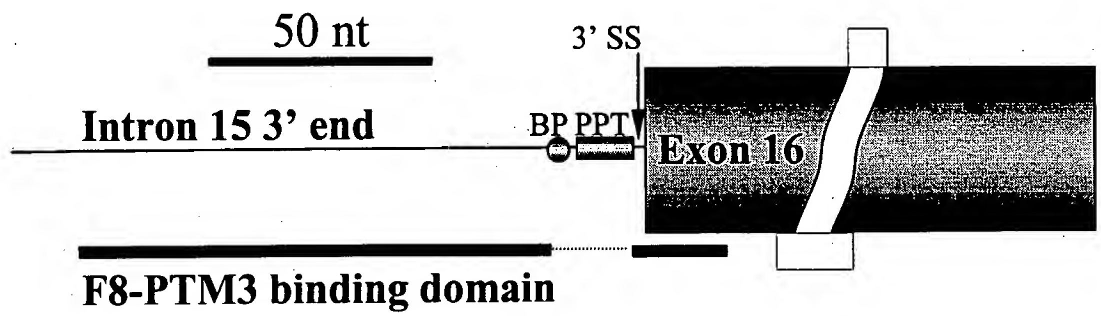


Figure 44 B

Figure 44C

NcoI
CCATGGTCGACGTTAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATT

TTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGCGCGCGCCAGGC

GGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGC$\boxed{\text{CAATCA}}$GAGCGGCGCG

CTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCC$\boxed{\text{TATAAA}}$AAGCGAAGCGCGCGGCGGGCG

F13 XhoI
$\boxed{\text{GGAGTCG}}$CTGCGACGCTGCCTTCGCCCCGTGCC**AACC**CTCCGCC**TCGAGCTTACCTGAACTAATTTTTTAGAA**

HindIII
TATTAAAATCCTAAGCTTTTATATCTCTATCCCTCTATCTTTTGCTCTCTATCCAATTTTTATTAACTTAGA

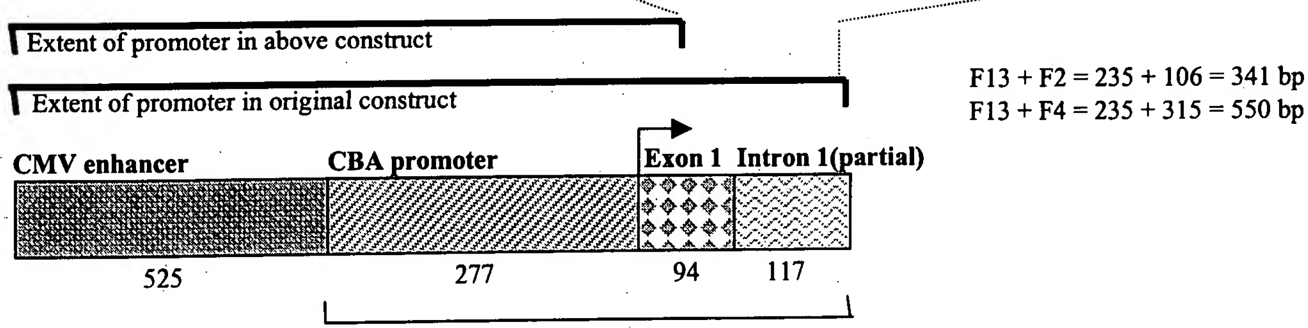
SacII
CTTTAAAAAGAAACTTATGAGAAAAATTT*CCGCGGAACATTATTATAACGTTGCTCGAATACTAACTGGTAC*

EcoRV PstI
CTCTTCTTTTTTTTTTGATATCCTGCAG

Chicken β-actin Promoter

Nucleotide changes are shown in blue
Boxed = CAT box, TATA box
Boxed + Arrow = Transcription Start
Oval = Downstream elements
Bold = Binding domain
Italicized = Spacer+PPT+BP+AG dinucleotide

<u>Sequence not included in construct</u>
CGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAG
CGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTAATGACGGCT
TGTTTCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCTCCGGGAGGAATTCGTA



F13 + F2 = 235 + 106 = 341 bp
F13 + F4 = 235 + 315 = 550 bp

Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

B4, P72

(Sheet 61 of 66)

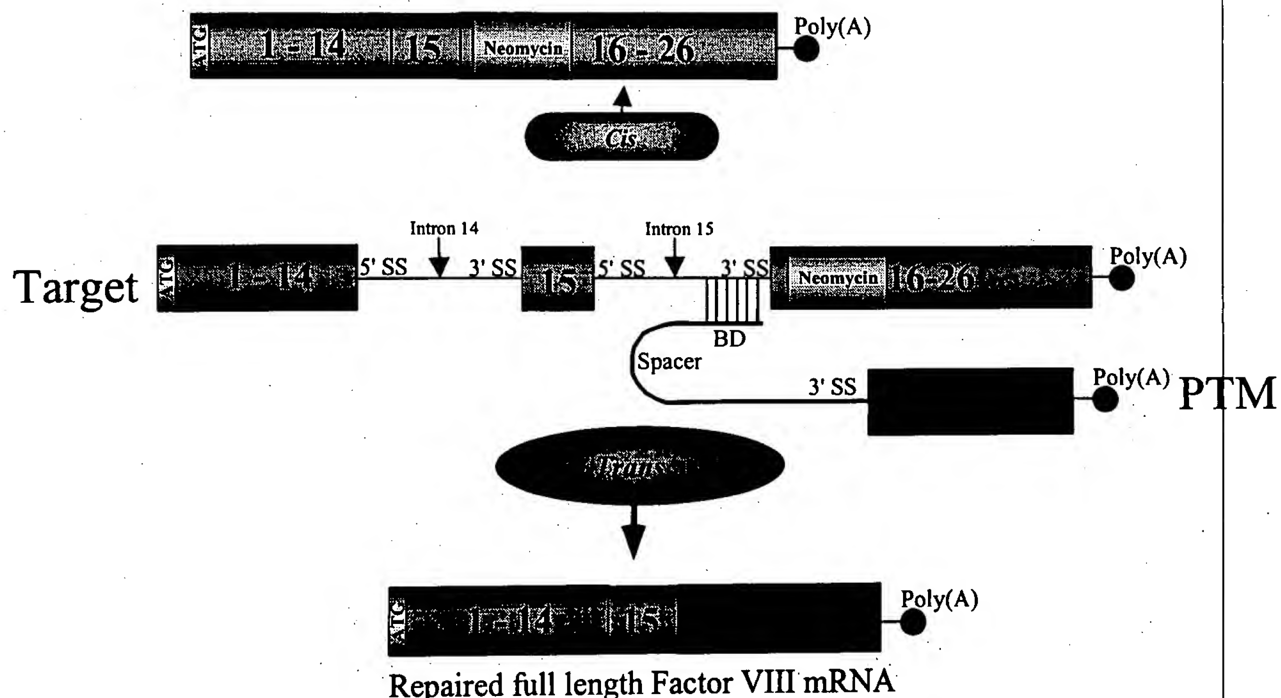
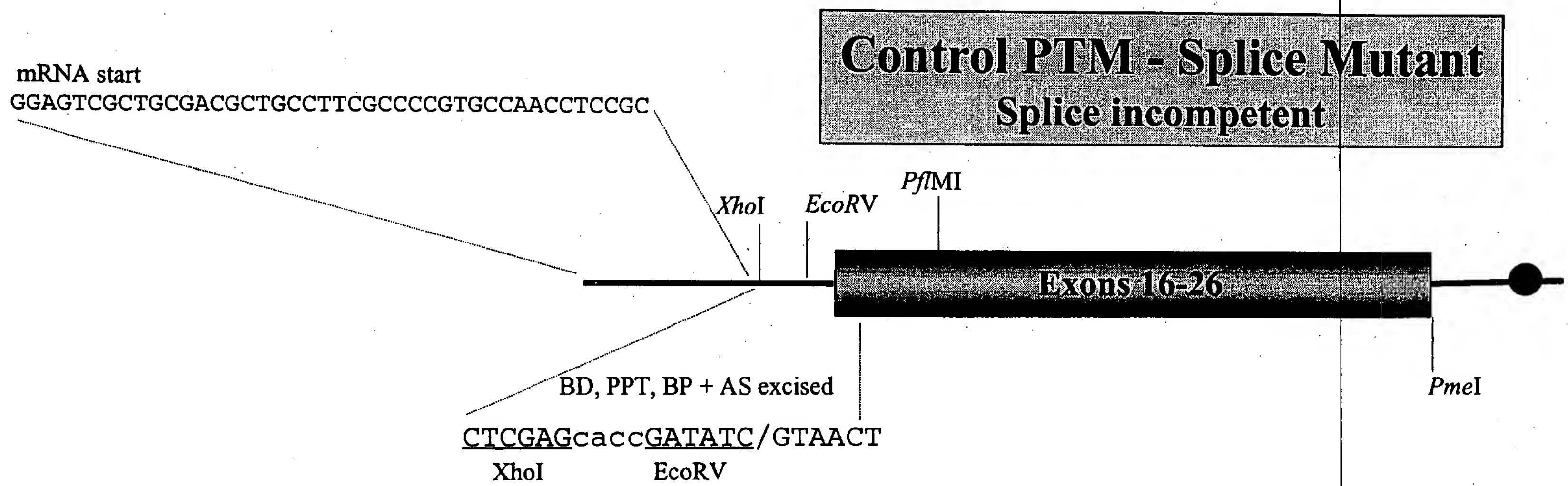

ATC
1 - 14
15
Neomycin
16 - 26
Poly(A)
Cis
Intron 14
Intron 15
Target
ATC
1 - 14
5' SS
3' SS
15
5' SS
3' SS
Neomycin
16-26
Poly(A)
BD
Spacer
3' SS
Poly(A)
PTM
Trans
ATC
1-14
15
Poly(A)
Repaired full length Factor VIII mRNA

Figure 44D

Figure 45

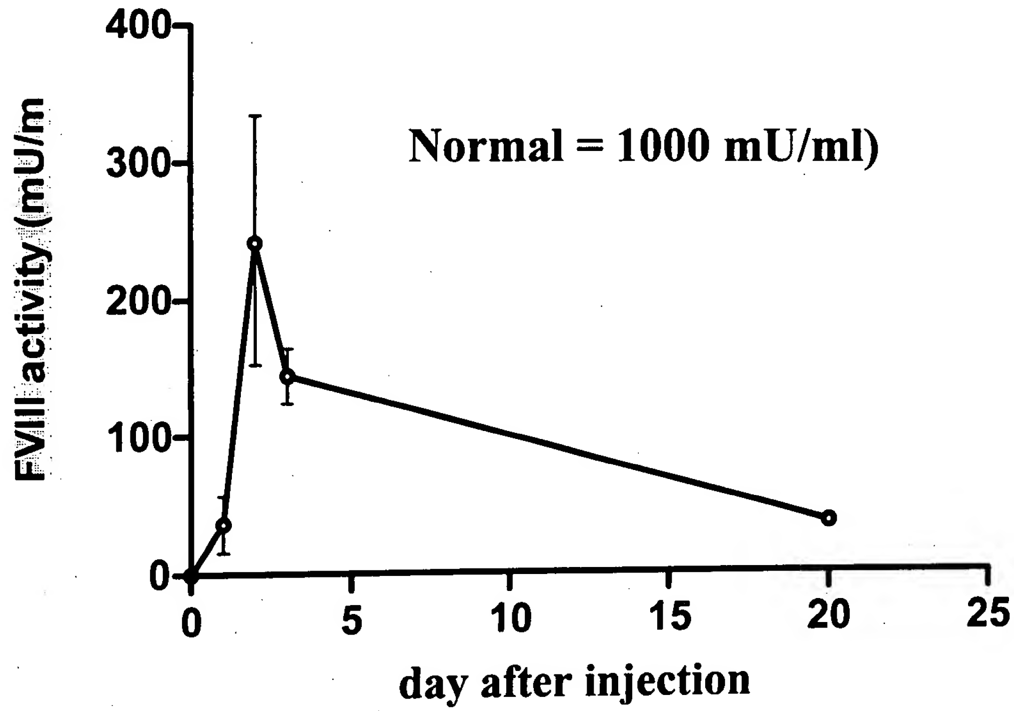


Method:
Excise TSD and part of exon 16 with
XhoI and PflMI and ligate in a PCR product that:
1) eliminates the TSD and splice acceptor site
2) inserts EcoRV adjacent to exon 16
3) restores the coding for exon 16

Repair of Factor VIII

Preliminary results from one experiment

FVIII activity in Exon 16 FVIII-KO mice after IV PTM-FVIII intraportal infusion (100ugDNA)(n=3)



METHODS

Inject plasmid intraportally

↓

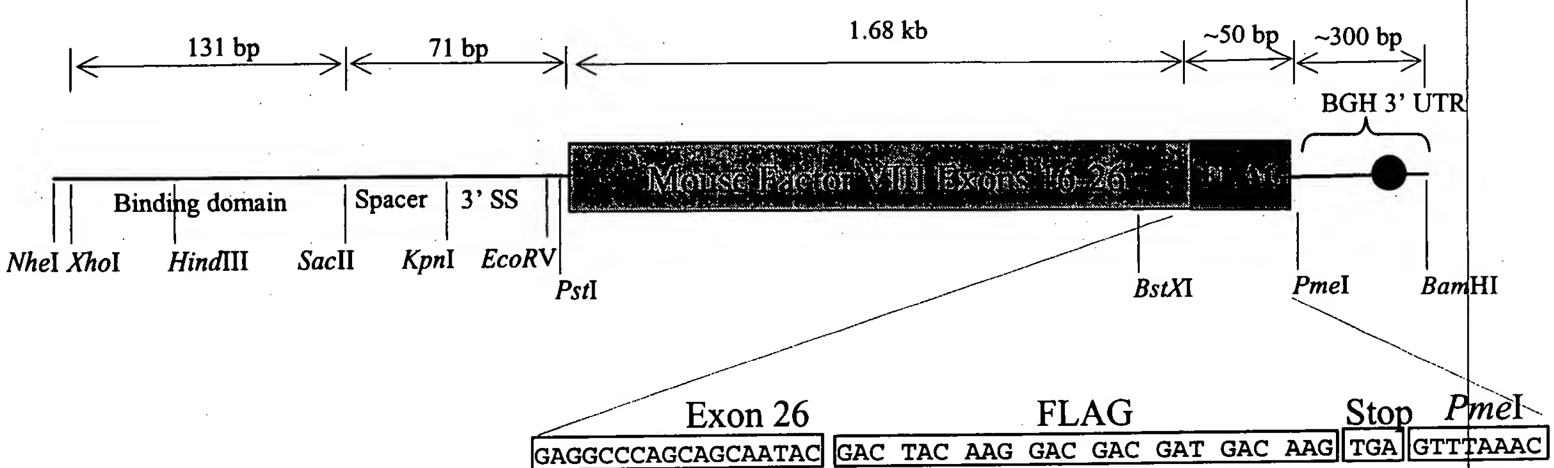
Sample blood (1, 2, 3, 20 d)

↓

Assay for factor VIII activity

Figure 46

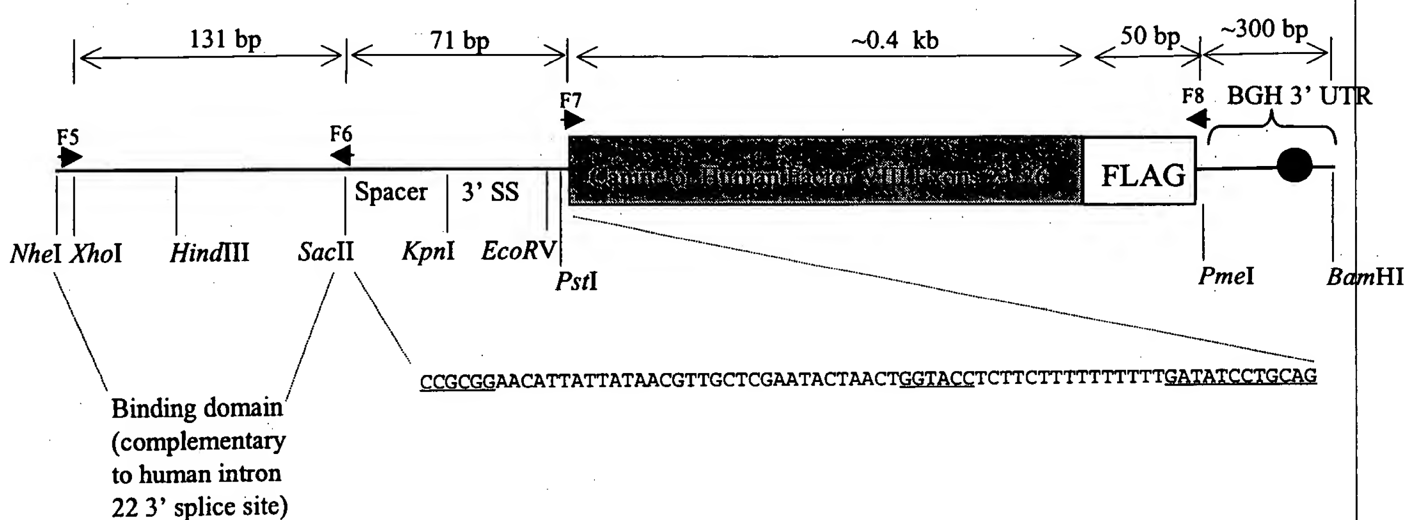
Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26 and a C-terminal FLAG tag. BGH = bovine growth hormone 3' UTR; Binding domain = 125 bp.



REFERENCE FOR DESIGN OF FLAG TAG

Brann T, Kayda D, Lyons RM, Shirley P, Roy S, Kaleko M, Smith T.
Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates.
Hum Gene Ther 1999 Dec 10;10(18):2999-3011
Genetic Therapy, Inc., a Novartis Company, Gaithersburg, MD 20878, USA.
Epitope-tagged B domain-deleted human factor VIII cDNA (flagged FVIII) was evaluated in nonhuman primates.

Figure 47A



FLAG = C-terminal tag to be used to detect repaired factor VIII protein.

Figure 47B